IN VITRO STUDIES ON *THEILERIA*-INFECTED LYMPHOBLASTOID CELL LINES

by

FRED LUSONZI MUSISI
B.V.M. (Nairobi) M.Sc. (London)

Thesis presented for the degree of
Doctor of Philosophy
of the University of London
in the Faculty of Medicine

London School of Hygiene and Tropical Medicine

APRIL 1979

In vitro studies on *Theileria*-infected lymphoblastoid cell lines

by Fred Lusonzi Musisi

ABSTRACT

A review of literature of the history of the development of *Theileria*-cell culture and studies on *Theileria*-infected lymphoblasts was carried out. A need for a systematic study of characteristics that might help identification of *Theileria* cell-lines as well as the relationship between *Theileria* and the host-cell *in vitro* was noted. As a result, a study of the characteristics of *Theileria*-infected cell-lines and the relationship between *Theileria* and the host lymphoblast cell was undertaken. Five cell-lines isolated by the author in Kenya (one infected with *T. lawrencei* in buffalo cells; three infected with *T. lawrencei* and one with *T. parva* in cattle cells) together with four cell-lines (one with *T. parva* and three with *T. annulata* in cattle cells) previously isolated by workers in East Africa and Iran were used in the study.

Electron microscopic studies on the relationship of *T. annulata*, *T. parva* and *T. lawrencei* with the host-cell revealed a close association of the parasite with the host-cell in that the host-cell treated the parasite in a way similar to its own nucleus by forming nucleopore-like structures with the parasite membrane, and during host-cell division the parasite was aligned along the mitotic spindle fibrils very much in the same way as the host-cell chromosomes

were. A very active Golgi apparatus was observed in all Theileria-infected lymphoblasts. No ultrastructural differentiating features between T. parva, T. lawrencei and T. annulata parasites could be found in the Theileria-infected lymphoblasts maintained in vitro.

Studies on isoenzyme variants between the various Theileria-infected cell-lines revealed only two enzymes that could be distinguished on a Theileria-species basis. These were glucose phosphate isomerase and glyceraldehyde phosphate dehydrogenase. The importance of the isoenzyme results is discussed.

There were no significant differences between all cattle Theileria-infected cell-lines with regard to percentages of cells parasitized, cells in mitosis and multinucleate cells or the mean schizont nuclear number. There were, however, significant differences between cattle Theileria-infected cells and buffalo Theileria-infected cells. The significance of these findings in relation to characterization of cell-lines is discussed.

CONTENTS

CHAPTER 7

CHAPTER 1

INTRODUCTION

1.1 *Theileria* parasites

Theilerias are protozoa whose biology and taxonomic status have posed problems ever since they were first associated with outbreaks of a highly fatal disease in Rhodesia and South Africa at the turn of this century. They are tick-borne; their worldwide occurrence in domestic stock and wild animals is well documented in reviews by Barnett (1977, 1968), Burridge (1975), Brocklesby and Vidler (1966) and Neitz (1957).

A number of specific names have been conferred which may not be valid. Basically the accepted specific names of *Theileria* affecting cattle are as follows: *Theileria parva*, *Theileria lawrencei*, *Theileria annulata*, *Theileria mutans* and *Theileria sergenti*. A detailed discussion of various names of *Theileria* parasites affecting cattle is, however, given in the general view of literature of the thesis but for ease of following older literature all the parasites are regarded as species although in some cases there may be no justification for so regarding some.

The distribution and names of the diseases caused by the parasites are summarized in Table I.

T. parva infection became known as ECF after the introduction of cattle, carrying the disease, from East Africa into Rhodesia and South Africa. Studies by Neitz et al. (1955) indicated that ECF was not a simple entity and that Rhodesian theileriosis was caused by a parasite originating from buffalo which was found to be killing cattle in a corridor of land between game reserves in South Africa. It therefore became known as Corridor disease and the causal agent was named T. lawrencei. Although T. mutans infections were called benign bovine theileriosis, it is now known that there are some which are not benign and a full discussion on this will be found in the general review of literature. In this thesis, the term theileriosis is used as a collective name describing infections due to one or all of the abovementioned parasites.

1.2 Importance of Theileria spp. affecting cattle

Losses due to these parasites arise from several factors. Direct losses can occur as a result of deaths attributable to either ECF or corridor disease, or tropical theileriosis. Further direct losses can be due to sub-clinical disease, leading to unthriftness and therefore to poor production. The latter losses are mainly observed with either T. mutans or T. sergenti infections.

Secondary losses arise from quarantine, dipping and reliance on less productive breeds of cattle in Theileria-enzootic areas. Imposition of a quarantine

means that the farmer has to pay for extra maintenance during this period. In enzootic areas, dipping to be effective has to be done twice weekly to avoid the risk of infecting cattle with ticks; this is a big financial drain in terms of purchase of acaricides, building dips or spray races and the time spent supervising dipping. Further losses occur due to development of resistance to acaricides. This may mean purchase of new and more expensive acaricides. Due to inadequate tick control there is retardation of agricultural development in many areas of Africa, because inhabitants of such areas have to rely on breeds of cattle which are relatively non-productive, but which nevertheless seem to be ECF-tolerant.

Eradication of ECF in South Africa was achieved through use of very stringent measures at an estimated cost of £50 million. The measures included slaughter of the affected animals and extensive application of acaricides. Most areas in the world where theileriosis is a problem are not in a position to afford similar costs.

1.3

Research project on tick-borne diseases

The United Nations Development Programme (UNDP) in conjunction with the Food and Agriculture Organization (FAO) published a report (Anon., 1967) in which it was concluded "In East Africa the tickborne diseases are serious killers of cattle and control of these diseases, above all East Coast Fever, deserves the highest priority for disease research." Consequently Kenya, Tanzania and Uganda received assistance from the UNDP through the FAO to set up a tick-borne disease

research project. The project was based at the then East African Veterinary Research Organization (EAVRO), Muguga, Kenya and the terms of reference were to investigate the feasibility of producing a vaccine against ECF. The author of this thesis joined the project as an East African Community Veterinary Research Officer (trainee), counterpart to FAO personnel.

Research by the project indicated that two approaches to immunization against ECF could be feasible: one was by infection and treatment (immunochemoprophylaxis) and the other by inoculation of attenuated *in vitro* propagated *Theileria* schizonts. A full discussion on various means of immunization is in the review of literature. However, despite the recognition of the feasibility of immunization, problems due to strain or species differences became a major stumbling block in attempts to achieve an effective and practical field immunization.

1.4

Aims of studies carried out for this thesis

The author set out to study the characteristics of *in vitro* propagated *Theileria*-infected lymphoblasts in the hope that findings from such studies might augment efforts being made to produce an effective cell culture vaccine. The objectives of the study were as follows

(a) to establish in culture *Theileria*-infected cell lines from experimentally infected cattle and naturally infected buffaloes, and then to study

(b) characteristics of these cell lines,

(c) the fine structural relationship between Theileria and lymphoblasts, and finally

(d) to compare electrophoretic mobilities of a selected number of enzymes from the cell lines infected with different species of Theileria with a view to distinguishing Theileria species.

1.5

Terminology

Macroschizont: this is the lymphocytic stage of the parasite in the vertebrate host which on Giemsa staining is characterized by presence of irregular big red or purple nuclei dispersed throughout the pale blue cytoplasm of the parasite. This stage is referred to as Koch's blue bodies or agamont in some older literature. Macroschizonts vary in size and shape and may range from 2 to 16 µm in diameter. The term macroschizont is misleading since it implies that there are two types of schizonts. It is, however, used in this thesis for ease of following older literature although schizont is the accurate term; schizont and macroschizonts have been used interchangeably in this thesis.

Microschizont: this is really a collection of merozoites in the lymphocytic cytoplasm and is characterized by presence of numerous very small, approximately uniform-sized, purple staining nuclei with a very small amount of cytoplasm. The particles are usually arranged at the periphery of the

parasite's cytoplasm and a residual body from which they originate may be present in the middle of the parasite mass. Cells containing this stage readily rupture to free the particles. Microschizonts have sometimes been called gamonts.

Merozoite: this is a product of the division of the microschizont stage. It is uninucleate, about 1 µm in length and consists of a round nucleus and a small amount of cytoplasm. This stage is sometimes referred to as the micromerozoite, a term which is misleading since it implies there are two types, a macromerozoite and micromerozoite.

Piroplasm: as applied to Theileria, refers to the intra-erythrocytic stage of the parasite. It can be comma-shaped, oval or round and measures between 1.2 to 2.5 µm in length. This stage has also sometimes been called the endoglobular parasite. Other piroplasms exist, for instance, Babesia piroplasms, but these are usually bigger than Theileria piroplasms.

Piroplasmosis: this was a general term applied to babesiosis and theileriosis mainly because at the turn of this century all the various intra erythrocytic stages of these diseases were believed to belong to the same organism.

Infective particle: this is the mature form of Theileria emanating from the tick salivary glands during tick feeding. It has also been called a sporozoite by Cowdry and Ham (1932) and Wilde et al. (1968).

In this thesis, the definitions given for primary isolate, stock, strain and line are based on proposals by the World Health Organization (WHO)(Anon., 1978) for salivarian trypanosomes.

Primary isolate: the viable organisms present in a cultural or in an experimental animal host following the introduction of a sample or part of a sample from a naturally infected host. It is noted that the primary isolate may consist of a mixture of species or subspecies.

Stock: the population derived by serial passage in vivo and in vitro from a primary isolate without any implication of homogeneity or characterization. Stock derived at different times from a single isolate may differ.

Strain: a set of populations originating from a group of organisms of a given species or subspecies present at a given time in a given host or culture and defined by possession of one or more designated characters.

Stock and strain: in this thesis stock is used instead of Strain because Strain is obtained by characterization of a subdivision of a Stock, and unambiguous characterization can be ensured only if such a subdivision is imitated by a single

organism. In literature on Theileria it is not at all clear whether single organisms were used to initiate what has been referred to as Strains or whether it is Stock which is being called Strain.

Line: a laboratory derivative of a stock maintained in different physical conditions or in different geographical locations. Different physical conditions includes different animal species as well as cultures.

Cell line : in this thesis, the term refers to both uninfected cells and cells infected with Theileria derived from a primary isolate and which have apparently developed the potential to be subcultured indefinitely in vitro.

Stabilate: refers to a sample of organisms viably preserved on a single occasion (Lumsden and Hardy, 1965). With Theileria stabilates can be either of infective particles (sporozoites) from the tick or of infected lymphoblastoid cells maintained in vitro.

CHAPTER 2

GENERAL REVIEW OF LITERATURE

2.1
Early History

Koch (1897) while investigating babesiosis in cattle near Dar-es-Salaam, Tanzania, found minute rod-shaped, oval, or round organisms in erythrocytes of some cattle. He thought they were young forms of Babesia bigemina. However, Koch (1903), Theiler (1903) and, Stephens and Christophers (1903) studied the cause and transmission of outbreaks of Rhodesian tick fever in Rhodesia and South Africa and concluded that the causal agent was the same as that described earlier by Koch in Tanzania. Stephens and Christophers (1903) proposed the name Piroplasma kochi while Theiler (1904) called it P. parvum. Koch (1906) described the lymphocytic stage of the organisms. Gonder (1910) conclusively showed that the lymphocytic stage also called Koch's blue bodies was a phase in the development of Theileria.

About the same period, Dschunskowsky and Luhs (1903) found similar organisms in erythrocytes in blood smears of Southern Russian cattle dying of a disease they called tropical piroplasmosis. In contrast these organisms were predominantly round and were called by Dschunskowsky and Luhs (1904) P. annulatum. Further studies by the same workers (1909) revealed the lymphocytic stage of the parasite.

Theiler (1906) described another organism responsible for a benign form of bovine piroplasmosis in South Africa

and called it P. mutans. It was distinguished from P. parvum by the fact that it had no lymphocytic stage. However, Theiler and Graf (1928) demonstrated the lymphocytic stage of P. mutans and this complicated differential diagnosis of Rhodesian tick fever. Consequently, differential diagnosis had to take into account epidemiology and clinical signs.

As a result of this complicated situation Lawrence (1933-34) attributed to P. mutans outbreaks of a serious mortality which occurred among cattle under ranching conditions in Rhodesia. He thought that resistance to P. mutans had broken down allowing the organism to develop rapidly in lymphocytic tissues. It is not clear why he chose P. mutans instead of P. parvum which was believed to be the lethal organism at the time. Probably the clinical signs and epidemiological observations did not fit the P. parvum picture. Neitz et al. (1953) encountered a disease with identical symptoms, in a corridor of land, a stretch of 100 square miles lying between Hluhluwe and Umfolozi game reserves. The disease occurred when cattle were introduced into the area; 300 of 585 animals died within a month. Mortality ceased within three weeks when cattle were removed to areas not frequented by buffaloes. Neitz et al. (1955) showed that Rhipicephalus appendiculatus was the vector of the causal agent. Neitz (1955) concluded that buffaloes could serve as a reservoir for infection of the ticks and that the organism was neither P. parvum nor P. mutans; he called it Gonderia lawrencei and the disease was called Corridor disease.

2.2

Taxonomy

2.2.1

Higher taxa:

Theileria belong to phylum protozoa but views on placement into Subphylum, Class, Order and Suborder have been varied. Wenyon (1926) gave the following classification:

Class:	Sporozoa
Order:	Coccidiida
Suborder	Piroplasmidea
Family:	Theileriidae
" :	Babesiidae

Neitz and Jansen (1956) proposed that a new Suborder of Leucosporidea be created to include family Theileriidae and other organisms closely resembling Theileria in morphology and life cycle. Suborder Piroplasmidea would then be left with one family Babesiidae. The proposed change was based on the observation that Babesia unlike Theileria, did not invade leucocytes and did not multiply by schizogony.

Since then there have been many suggestions on the placement of Theileria and Babesia in the higher taxa. To illustrate the confusion that has existed, and the changing views on classification of these organisms, Brocklesby (1978) gave Table II. The table which does not quote references, nevertheless emphasizes the point that classification of Theileria and Babesia has been an unresolved issue for a long time. Over the last seven years there has not been much published on classification of Theileria and inclusion of the parasites in subphylum Apicomplexa (Levine, 1970,1971) may not be the final solution for classification.

2.2.2

Family and genus

Table III summarizes changing views on classification of various Theileria spp. at family and genus level. It shows that problems of classification were not confined to those of placement in the higher taxa. It is only in the last fifteen years that the various Theileria spp. affecting cattle have been firmly accepted as belonging to the family Theileriidae.

Originally Babesia and Theileria erythrocytic forms were under the same genus of Piroplasma until Bettencourt (1907) separated them (Table III). Genus Gonderia was proposed by Du Toit (1918) to include piroplasms whose stages resembled those of Theileria parva but whose lymphocytic stage had not been demonstrated while Neitz and Jansen (1956) proposed family Gonderidae with genus Gonderia to distinguish both T. annulata and T. mutans which multiplied in erythrocytes from T. parva which did not (Table III). However, Neitz (1963) declared that both family Gonderidae and genus Gonderia were invalid. No reasons were given. This invalidation might have been a result of discovering that T. parva could also maintain itself in cattle erythrocytes in the absence of demonstrable schizonts (Neitz 1964).

2.2.3

Species

There is also controversy on speciation within the genus Theileria. Until recently Theileria spp. affecting cattle in East Africa were considered to be T. parva Theiler (1904), T. lawrencei Neitz (1955) or T. mutans Theiler (1906). However, Brocklesby (1966) provided evidence challenging the validity of T. lawrencei and supported Du Toit (1930) who stated that all species of Theileria infecting cattle were strains of T. parva. Brocklesby (1966) gave the following classical criteria (Table IV) for differentiating T. parva from T. lawrencei.

However, Brocklesby (1966) found an increase in macroschizont size and numbers, and an increase in microschizont and piroplasm numbers when a Kenyan strain of T. lawrencei was passaged through cattle. This meant that the parasite had become indistinguishable from T. parva. He also found similar changes on passage of a South African stock to T. lawrencei. Young and Purnell (1973) reported similar observations on a Theileria species, pathogenic to cattle, which was isolated from a buffalo in the Seregenti National Park, Tanzania. These are the only two reports of transformation of T. lawrencei into a T. parva-like parasite. Levine, 1971

classified T. lawrencei as a synonym of T. parva. However, in this thesis, T. parva and T. lawrencei are treated as separate species on the basis of cross-immunity studies reported by Schindler et al. (1969). Young et al. (1973), Radley et al. (1975) and Burridge (1975).

In Europe, North Africa and Asia, distinct names were given to parasites in different countries, for instance T. dispar in Algeria (Sergent et al. 1924), T. turkestanica in Turkestan (Oboldoueff and Galouzo, 1928) and T. sergenti in East Asia (Yakimoff and Dekhetereff, 1930). Nothing was known of the biological transmission and immunological relationship of these parasites to T. annulata and T. parva. Neitz (1957) reported that following cross-immunity studies by various workers, T. dispar, T. annulata and Theileria stocks in Russia were shown to be antigenically related and transmitted by various Hyalomma spp. T. dispar, T. turkestanica and T. sergenti were therefore regarded as synonyms of T. annulata.

With regard to T. mutans, the name has been applied to cattle Theileria of low pathogenicity in many parts of the world ever since it was described by Theiler (1906). Uilenberg et al. (1978) reported that the South African and East African T. mutans were serologically identical but different from the British and Australian T. mutans. The British and Australian parasites are serologically related but morphologically different from the African T. mutans. Morzaria et al. (1977) noted that no schizonts have been reported in tick-induced infections of the Australian, British and Japanese cattle Theileria but schizonts are usually present

in African T. mutans infections. The validity of the names applied to the Japanese cattle Theileria is questionable since Neitz (1957) in a review on theileriosis, gonderiosis and cytauxzoonoses indicated that T. sergenti Yakimoff Dekhtereff (1930) was a synonym of T. annulata whereas Barnett (1968) and Ishihara (1968) have suggested that cattle Theileria in Japan described as T. mutans might, in fact, be T. sergenti Yakimoff and Dekhtereff (1930). Barnett (1968) and Brocklesby (1978) are of the opinion that there are four valid cattle Theileria spp. and these are T. parva, T. annulata, T. mutans and T. sergenti.

2.3
The life cycle of Theileria spp.

Figure 1 summarizes the life cycle of Theileria spp. as it is known to date. Most of the stages are intracellular. The free stages are indicated on the diagram.

2.3.1
Life cycle in the vertebrate host:

Radley et al. (1974) proposed that there might be a stage between the infective stage from the tick and the uninucleate lymphocytic stage preceding the macroschizont stage. This speculation was based on failure to demonstrate parasites until 5 days after inoculation of infective particles into cattle. Increasing doses of infective material failed to reduce prepatent period below 5 days. One explanation given for failure to demonstrate parasites during this

time was that the parasite could be disguised in a form not capable of being stained by the Giemsa method.

Macroschizonts and microschizonts develop in local lymph nodes in the area where inoculation of infective particles is effected. They later appear throughout other lymphoid organs in the body and sometimes, in non-lymphoid organs such as heart muscle. The mechanism of spread of parasitized lymphocytes is unclear but there are indications that tracheal and thoracic lymph ducts could play a part (de Martini and Moulton, 1973), possibly by discharging these lymphocytes into the jugular vein. The parasitized cells could then enter tissues by peripheral arteriolymphatic communications or through post-capillary venules.

Mode of multiplication of macroschizonts was unclear until Hulliger et al. (1964) provided evidence that macroschizonts were split into two during host cell mitosis and were distributed into daughter cells. Zablotskii (1967) and Malmquist et al. (1970) confirmed these observations. These observations were made on infected lymphoblasts maintained in culture but Reichenow (1940), de Martini and Moulton (1973) and Young et al. (1978c) noted similar mode of multiplication in cells in vivo. This seems to be the only mode of multiplication of macroschizonts although Gonder (1910) and Cowdry and Danks (1933) were of the opinion that Theileria multiplied by schizogony in lymphocytes and produced merozites to infect other lymphocytes and erythrocytes.

Macroschizonts later transform into microschizonts but the trigger for this change is not known. Microschizonts release merozoites which invade erythrocytes to produce

piroplasms. Jarrett et al. (1969) and Radley et al. (1974) observed that appearance of piroplasms was time dependent and not dose dependent. Jarrett et al. (1969) interpreted this as an indication that Theileria undergoes a fixed number of replications in lymphocytes before maturing into micro-schizonts and piroplasms.

2.3.2
Life cycle in the tick: (Figure 1)

2.3.2.1
Development in the lumen and haemolymph

Developmental stages in the intestinal lumen, intestinal epithelial cells and salivary glands of ticks were described by Cowdry and Ham (1932) for T. parva but Reichenow (1940) refuted their findings, except for some stages in the salivary glands, on the grounds that he could observe similar structures in uninfected ticks. However, studies of developmental stages of T. annulata (Schein, 1975) and T. parva (Schein et al., 1977) produced results which generally agreed with the findings of Cowdry and Ham (1932). The part of the life cycle of Theileria in the tick in Fig. 1 is based on results reported by Schein (1975), Schein et al. (1977) and Purnell and Joyner (1968).

Schein (1975) and Schein et al. (1977) observed two forms of the parasite in the lumen of the gut; a large and a small form. The small form became elongated and sometimes developed spike like processes, but finally developed into thread-like parasites resembling microgametes of Plasmodium spp. The larger form had a cloudy cytoplasm but

did not undergo the development described for the smaller form. Schein (1975) considered the small form a microgamete and the larger a macrogamete and assumed that the two fused to form a zygote although no syngamy was actually observed. The assumption was based on the fact that only one developmental form could later be detected in the epithelial cells of the gut. This form had nuclear material on the edge and cytoplasm in the centre. It increased in size and later migrated from epithelial cells through haemolymph and into salivary gland acinar cells of the tick.

2.3.2.2
Development in the salivary glands:

Sporogony of the salivary gland stage of the parasite occurs after moulting of the tick. Purnell and Joyner (1968) recognized three forms of the parasite which they designated 'young' 'intermediate' and 'mature' forms. These are the different developmental stages during sporogony. With Giemsa stain, young forms stain pale blue and lack nuclear differentiation while the intermediate forms occur as a mass with blocks containing varying nuclear material with or without clusters of nuclear material. Mature forms, also called infective particles, were numerous, small and discrete with pale blue staining nucleus.

Purnell and Joyner (1968) could detect Theileria in unfed ticks although Martin et al. (1964) and Reichenow (1940) could not. Although feeding on an animal by the tick was thought necessary for maturation of the infective parasites, Samish (1977) showed that a temperature of 37^{o}C with relative humidity of 95% for 6 days was enough.

A similar mode of development in tick salivary glands has been observed for T. lawrencei by Young et al. (1975) and for T. mutans by Purnell et al. (1975). This therefore is likely to be a common process of development for all Theileria spp.

2.4 Clinical disease and diagnosis

Infections with either T. parva, T. lawrencei, T. annulata or T. mutans result in a lymphoproliferative disease clinically manifest as swollen superficial lymph nodes. Lymph node biopsy smears reveal macroschizonts one to two days following onset of the swelling (Barnett, 1960; de Martini and Moulton, 1973; De Kock, 1957). Fever follows a few days after appearance of the macroschizonts and is usually persistent until death or recovery but Young et al. (1978) have reported that fever does not seem to be a regular feature for T. mutans infections.

2.4.1 Features peculiar to T. parva and T. lawrencei infections

The main clinical features peculiar to T. parva infections (and usually to T. lawrencei infections) are leucopenia, petechial haemorrhages and pulmonary oedema. Leucopenia is a result of extensive prolonged cell destruction and arrest of maturation of lymphoreticular tissue cells which follow a transient development and proliferation of all cell types (Barnett, 1960). Kimeto (1976) studied

ultrastructural changes of thrombocytes in cattle with ECF and found vacuolation, degranulation and thrombocytolysis as the major features. These are now believed to be responsible for the widespread petechial haemorrhages in mucuous membranes of diseased cattle. De Martini and Moulton (1973) have suggested that pulmonary oedema and dyspnoea might be related to accumulation of parasitized lymphocytes in the lungs and the associated (bronchial and mediastinal) lymph nodes. Barnett (1960), and De Martini and Moulton (1973) found these lymph nodes more severely affected than other nodes in the body. Pulmonary oedema could also be partly due to platelet degranulation and serotonin release. The pharmacological action of serotonin is to produce increased vascular permeability and oedema (Maling et al., 1974).

2.4.2

Features peculiar to T. annulata and T. mutans infections

Although T. annulata infection is equally lymphoproliferative there is a greater focus on the liver, so much so that jaundice is a regular feature. Liver biopsies are routinely taken much with the same frequency as lymph node biopsies are in T. parva and T. lawrencei infections. Barnett (1960) and De Kock (1957) have observed that liver tissue does not appear to be severely affected in T. parva infections.

Anaemia is very pronounced in T. annulata (Neitz, 1957) and in T. mutans (Young et al. 1978, Neitz, 1957) infections. It is the most consistent feature for T. mutans but hardly a major clinical feature in T. parva or

T. lawrencei infections. Anaemia might reflect the ability of T. annulata and T. mutans to multiply within erythrocytes and invade fresh erythrocytes - a feature reported by Pipano (1976) and Young et al. (1978) but very uncharacteristic of T. parva and T. lawrencei piroplasms. Nevertheless, Neitz (1964) investigated the ability of T. parva and T. lawrencei to multiply within erythrocytes and invade other erythrocytes and concluded that they did so. If observations by Neitz (1964) were correct then it might be the degree of multiplication within erythrocytes that determines the extent to which anaemia manifests itself.

2.4.3

Diagnosis

Diagnosis of the disease and parasite involved therefore depends on the clinical features and parasitological examinations of Giemsa stained blood, lymph node or liver biopsy smears. Serology and epidemiological knowledge can help in determining which parasite species is involved in some cases.

2.5

Treatment

Neitz (1957) gave exhaustive lists of drugs tried in the treatment of theileriosis. He reported that pamaquin had a selective action on the intraerythrocytic stages of Theileria but concluded that there was no single drug effective against the parasite during clinical disease. In a review on ECF, Wilde (1967) came to similar conclusions.

Pipano (1976) reported that no drugs active against T. annulata schizonts had been marketed.

However, Neitz (1953) had found that application of infected ticks to cattle followed by administration of tetracyclines over several days could protect cattle against a lethal challenge of ECF. Since then, attempts have been made to determine the chemotherapeutic value of tetracyclines on the overt disease. The latest report on this was by Brown et al. (1977) who showed that treatment with tetracyclines, if started immediately at the onset of patent ECF, can be significantly effective. However, this observation may not have practical value in the field since detection of onset of fever is not easy as it would entail monitoring temperature changes daily.

However, the search for an appropriate drug has continued and the latest addition to the list of drugs tested is Menoctone (2-hydroxy-3-(8-cyclohexyloctyl)-1,4-naphthoquinone). McHardy et al. (1976) found menoctone to be effective against macroschizonts of T. parva and T. annulata in vitro. They also found a high level of activity of menoctone against T. parva in experimentally infected cattle when the drug was injected on the first day of fever. Seven immunised cattle survived a challenge that killed six out of seven untreated control cattle. The usefulness of the drug appears to be vitiated by the impracticability of detection of onset of fever by a farmer otherwise it will only be of great use if it can be shown to be consistently effective in curing the disease several days after onset of fever and detection of macroschizonts.

2.6
Epidemiology and control:

2.6.1
Geographical distribution

T. parva and T. lawrencei are confined to the African continent South of the Sahara while T. annulata is widespread in countries bordering the Mediterranean Sea, the U.S.S.R., Iraq, Iran and India, and T. mutans is cosmopolitan. Although geographical conditions in some parts of West Africa are fairly similar to those elsewhere in equatorial and tropical Africa, T. parva and T. lawrencei have not been reported in West Africa. Barnett (1977) has suggested that T. lawrencei infections may not occur in West Africa because of lack of a suitable vector, R. appendiculatus. ECF is enzootic in Kenya, Malawi, Tanzania and Uganda but has occurred in epizootics in South Africa and Rhodesia (Neitz 1957) and has been reported in Mozambique, Ruanda, Zaire and Zambia but the extent to which the disease might be a problem in these countries is not clear.

2.6.2
Susceptibility and host range:

2.6.2.1
Age and breed of cattle affecting susceptibility

All cattle are susceptible to Theileria spp. described above but the degree of susceptibility may vary according to age and breed. Barnett (1957) investigated the effect of age on course of ECF and observed considerable resistance in cattle reared in enzootic areas. Resistance decreased

with age. However, recoveries at fourteen months of age suggested that genetic resistance played a part since passive colostral immunity would hardly persist for that long. Further support for susceptibility being related to age and possibly breed was provided by Guilbride and Opwata (1963) who found that cross-bred Jersey-Nganda calves were resistant to ECF during the first three months of their life but their resistance drastically dropped from the fourth month onwards such that mortality reached 100%. They attributed resistance in the first three months to maternal antibodies. In contrast to the crossbred calves, more than 90% Nganda (Bos indicus) calves resisted 10-infected tick exposure although when eventually introduced into a field with a severe ECF challenge they succumbed. Pipano (1976) also reported a degree of innate resistance in relation to breeds. He found dairy cows from Theileria-free areas to be most susceptible to T. annulata and when dairy and beef cattle imported from Theileria-free areas were immunized in a similar manner less protection was achieved in the dairy cows.

2.6.2.2

Role of wild animals in T. annulata infections

In a review of basic principles of T. annulata control, Pipano (1976) stated that cattle should be considered the main reservoir of infection and wild animals do not appear to play any role in the epidemiology of the disease. The role of the water buffalo in the epidemiology of T. annulata infections has yet to be clarified although Neitz (1957) cited literature to show that both water buffalo

with age. However, recoveries at fourteen months of age suggested that genetic resistance played a part since passive colostral immunity would hardly persist for that long. Further support for susceptibility being related to age and possibly breed was provided by Guilbride and Opwata (1963) who found that cross-bred Jersey-Nganda calves were resistant to ECF during the first three months of their life but their resistance drastically dropped from the fourth month onwards such that mortality reached 100%. They attributed resistance in the first three months to maternal antibodies. In contrast to the crossbred calves, more than 90% Nganda (Bos indicus) calves resisted 10-infected tick exposure although when eventually introduced into a field with a severe ECF challenge they succumbed. Pipano (1976) also reported a degree of innate resistance in relation to breeds. He found dairy cows from Theileria-free areas to be most susceptible to T. annulata and when dairy and beef cattle imported from Theileria-free areas were immunized in a similar manner less protection was achieved in the dairy cows.

2.6.2.2

Role of wild animals in T. annulata infections

In a review of basic principles of T. annulata control, Pipano (1976) stated that cattle should be considered the main reservoir of infection and wild animals do not appear to play any role in the epidemiology of the disease. The role of the water buffalo in the epidemiology of T. annulata infections has yet to be clarified although Neitz (1957) cited literature to show that both water buffalo

(Bubalus bubalis) and the American bison (Bison bison) were susceptible.

2.6.2.3

Role of wild animals in epidemiology of theileriosis in Eastern Africa

Wild animals susceptible to T. lawrencei infection are the African buffalo (Syncerus caffer) (Neitz, 1955; Kluge and Neitz, 1956; Young et al., 1973) and the Indian water buffalo (Burridge and Odeke, 1973). Both the African buffalo and the Indian buffalo have also been reported susceptible to T. mutans (Neitz, 1957). Similarly, T. parva infections have been reported in the African buffalo by Lewis (1943), Barnett and Brocklesby (1966) and in the Indian water buffalo by Neitz (1940).

The role of other bovidae in the epidemiology of ECF is not clear; the eland (Tautrotragus oryx), in particular, has been suspected of playing a part but unequivocal evidence is lacking. Brocklesby (1962) reported a fatal piroplasm infection in an eland in which schizonts were found in the liver, lung and lymph nodes but not in spleen or kidneys. Developmental stages of the parasite in the tick analogous to those of T. parva were seen in ticks collected from the dead eland. However, attempts to transmit the parasite were not successful in two out of three cattle but the third animal developed schizonts and piroplasms which were indistinguishable from those of T. parva or T. mutans. Attempts to transmit the infection to an eland calf resulted in lymph node enlargement but no parasites could be

demonstrated. A second attempt resulted in appearance of intra erythrocytic piroplasms but clean nymphs fed on the animal could not transmit the infection although the parasite could be demonstrated within the salivary glands of the resultant adults. Challenge of this eland with 50 infected ticks did not lead to theileriosis. These results were inconclusive since it was not clear whether the eland parasite was T. parva. Irvin et al. (1972b) fed R. appendiculatus and Rhipicephalus simus ticks on seven parasitaemic elands. Parasites were detected in the salivary glands of the ticks but attempts to transmit the infection to cattle failed. They concluded that the eland was unlikely to be implicated in the epidemiology of ECF.

Kimber (1974) studied serological relationships between E. African cattle Theileria spp. and Theileria isolated from game animals and found that a Theileria sp. isolated from an eland cross reacted with only Theileria sp. (Githunguri) (Burridge et al., 1974). Theileria sp. (Githunguri) was a cattle theilerial parasite, of low virulence isolated from cattle at Githunguri in Kenya but this parasite does not cross react serologically with either T. parva or T. lawrencei. Recently, Young et al. (1977b) have described a tick transmissible Theileria sp. isolated from elands infective for cattle but cattle recovering from it were fully susceptible to T. parva. The above information is inconclusive on whether the eland really plays a role in the epidemiology of ECF.

2.6.2.4.

Susceptibility of small laboratory animals

Despite the apparent widespread occurrence of theilerial parasites in game animals no small laboratory animal species appear to be susceptible. Brocklesby and Vidler

(1961) made several attempts to infect syrian hamsters (Mesocricetus auratus), rabbits (Oryctolagus cuniculus), multimammate rats (Rattus natalensis) and unstriped grass mouse (Arvicanthus abyssinicus) but none of these became infected. Irvin et al. (1976) inoculated in vitro propagated Theileria-infected lymphoblasts into mice, tumour-like masses developed but on karyotyping, parasites could only be found in cattle lymphoblasts and none in mouse cells.

2.6.3
Transmission

Under natural conditions, all cattle Theileria spp. described so far are transmitted by ticks. This transmission is transtadial. Although mechanical transmission of T. annulata and T. mutans intraerythrocytic stages can easily be effected by syringe passage it has not been reported to occur in nature.

T. annulata is transmitted by ticks of Hyalomma spp. Pipano (1976) noted that two ecological situations existed: the barn and the field. Hyalomma detritum is responsible for infection of cattle in barns while H. anatolicum and H. excavatum are responsible for infections in the field. They transmitted this parasite shortly after attachment to cattle. Battacharyulu et al. (1975) transmitted the infection within 24 hours of feeding of H. anatolicum while Samish and Pipano (1976) transmitted the infection within 2 to 3 days of feeding adult H. excavatum on cattle. These observations

indicate that control of the disease by dipping will be impractical.

Although several spp. of Rhipicephalus ticks have been shown to transmit T. parva experimentally, only R. appendiculatus plays a major role in ECF and Corridor Disease. Barnett (1977) observed that distribution of T. parva and T. lawrencei are so closely linked to the distribution of R. appendiculatus that the tick must be regarded as the only effective vector. Unlike T. annulata infections which are most common between July and September, there is no evidence of seasonal incidence for ECF in East Africa. This is presumably because adult vector ticks are present throughout the year due to occurrence of several generations in a year and therefore more or less a continuous infection is maintained throughout the year.

T. mutans is ubiquitous and is transmitted by different species of ticks in differents parts of the world. In South Africa Neitz (1957) reported R. appendiculatus as the vector but also noted that Theiler (1907) had transmitted the infection with Rhipicephalus evertsi. In contrast, Brocklesby, (1965), Purnell et al. (1970) and Irvin et al. (1972) were unable to transmit the infection with R. appendiculatus in Kenya. On the other hand, Uilenberg et al. (1978) and Young et al. (1978) have transmitted East African T. mutans parasites with Amblyomma variegatum. Young et al. (1977) transmitted a T. mutans-like parasite to cattle from buffalo using Amblyomma cohaerens ticks. Young et al. (1977, 1978) were of the opinion that A. cohaerens was the natural vector of T. mutans in E. Africa and also that Amblyomma gemma and Amblyomma lepidum might be vectors since they are common on

cattle in that part of the world.

In other parts of the world, Haemaphysalis spp. are the main vectors of T. mutans or T. mutans-like organisms. Brocklesby et al. (1975) reported transmission of T. mutans with Haemaphysalis punctata in the U.K. In Australia and Japan, Haemaphysalis longicornis transmits the infection but in addition Haemaphysalis bancrofti plays a role in Australia (Uilenberg, 1978). Brocklesby (1976) has reported that Russian and Japanese workers have transmitted what they call T. sergenti with Haemaphysalis japonicum. He reported that T. sergenti in this case is neither T. annulata nor T. mutans and this conclusion was said to have been based on serology and xenodiagnosis.

2.6.4
Morbidity and mortality

Morbidity and mortality vary from place to place and from time to time because they are a result of interaction of the following:

i) introduction of cattle from Theileria-free areas into Theileria affected areas
ii) density of vector ticks at a given time of the year
iii) species or strain of parasite involved
iv) concurrent infections like helminthiasis, anaplasmosis and babesiosis.

In cattle susceptible to ECF, morbidity rates of 87.6% (Brocklesby et al., 1961) and 93.1% (Wilde et al., 1968) were reported and both groups of workers were in agreement on a mortality rate of 95.5% of the infected stock. In the field, morbidity and mortality would be influenced by numbers of vector ticks such that a massive infected tick infestation would usually lead to high morbidity-mortality rates while low number tick infestation might lead to inapparent or mild disease irrespective of the virulence of the strain involved. In enzootic areas where there is a continuous tick challenge the exposure rate is very high and there is a stable situation between disease and host such that mortalities of below 10% are restricted to the new population of calves otherwise cattle are successfully raised in the presence of the disease (Barnett, 1977). In this case it is the recently introduced susceptible cattle that experience high mortalities.

Another important factor is the species or strain of parasite involved. In Africa, T. parva and T. lawrencei occur in the same areas but immunity to one does not necessarily protect the animal against infection from the other spp. (Cunningham et al., 1974; Schindler et al., 1969). As a result, when one of the species originally non-existent in the area is introduced there may be high mortalities and these might be misinterpreted as being due to the local species. With regards to strains in some areas of East Africa, where distribution of R. appendiculatus might be marginal but that of Amblyomma spp. widespread, morbidity and mortality due to pathogenic strains of T. mutans (Irvin et al., 1972, Young et al., 1978) might occur; the deaths

might then be attributed to ECF or Corridor disease because T. mutans is widely regarded as a non pathogenic organism.

2.6.5

Control

Despite high mortalities no effective treatment of theileriosis exists. Control of the vector ticks and/or immunization are the only alternatives. In African South of the Sahara, immunization is not yet a practical procedure. Vector control problems were highlighted in the introduction. The relatively short period within which ticks begin to inject infective particles following attachment on cattle render the present control procedures uneconomical and sometimes useless. In T. annulata areas some of these problems have been avoided by immunization of cattle.

2.7.

Immunology of immunization

2.7.1.

Antigens and antibodies

There are two types of antigens of practical importance; they are the schizont antigens in the lymphoid cells and the piroplasm antigen in the erythrocyte. Complement fixing antibodies, haemagglutinating antibodies and immunofluorescent antibodies in sera of infected or recovered cattle have been described for T. parva, T. lawrencei, T. annulata and T. mutans by various workers cited below.

2.7.2
Serological tests

Schindler and Wokatsch (1965) using either schizont or piroplasm antigens demonstrated that T. parva, T. annulata and T. mutans could be differentiated by the complement fixation test (CFT) and the indirect fluorescent antibody (IFA) test. Tutushin (1969) used the haemagglutination test to detect T. annulata antibodies and Duffus and Wagner (1974) showed that T. parva antibodies to piroplasm antigen could be detected by an indirect haemagglutination (IHA) test.

2.7.2a
IFA test

Although both the schizont and piroplasm antigen could be used, in practice only the piroplasm antigen was widely used in earlier studies because there are fewer schizonts in peripheral blood than piroplasms. In the case of T. annulata large numbers of schizonts could be obtained from liver while large numbers of schizonts for T. parva were obtained from spleens at autopsy. Whichever antigen was used, there was a problem of excessive background fluorescence. Lohr and Ross (1969) minimized the problem by washing parasitized erythrocytes in saline.

Further improvements of the test were made by Burridge and Kimber (1972) when they showed that schizonts from in vitro cultures gave specific fluorescence with sera from T. parva-infected cattle but no fluorescence with either conjugate alone or negative control serum. These authors also noted that significant IFA titres to schizont antigen

remained demonstrable for a longer period following recovery than IFA titres to piroplasm antigen. Use of cultured schizont antigen and the fact that patterns of antibody response studied using eluates of blood samples on filter paper were similar to patterns of antibodies in serum samples (Kimber and Burridge, 1972) have greatly facilitated and enhanced use of the IFA test in epidemiological studies. Of the tests mentioned above the IFA test is the only one that is widely used in the study of theileriosis.

2.7.3.

Immunity following natural infection

A recovery from natural infection from any one of these infections leads to immunity against a homologous challenge (Pipano, 1976; Neitz, 1957). Recovery from T. annulata and T. mutans results in a premunition type of immunity because low numbers of intraerythrocytic parasites remain detectable for years (Pipano, 1976; Neitz, 1964; Young et al., 1978). Recovery from T. parva or T. lawrencei is assumed to lead to a sterile immunity because no stage of the parasite is detectable; however Barnett (1977) has suggested that re-assessment of this is necessary. Since recovery is followed by protective immunity, attempts have been made to immunize cattle against these infections.

2.7.4

Artificial immunization against T. parva - T.lawrencei complex

Attempts to immunize cattle against this group of parasites can be divided into three approaches, namely, use

of the lymphocytic stage of the parasite, titration of infective particles from the vector tick so as to achieve inapparent or mild reaction to the infection and chemoprophylaxis.

2.7.4.1
Use of schizont stage from animals dying of *Theileria* infection

Attempts to use spleen and lymph node suspensions from infected cattle for immunization of susceptible stock were carried out by Spreull (1914). 25% of the cattle died as a result of the treatment; 70% of the survivors were partially or completely immune on challenge. Brocklesby *et al*. (1965) protected 15 out of 19 cattle inoculated with spleen suspensions from *T. parva* infected cattle against challenge of tick-induced infections. Pirie *et al*. (1970) inoculated cattle with 10^8, 10^9 and 10^{10} parasitized lymphocytes and found that fever only occurred in groups receiving 10^9 and 10^{10} cells and only one animal died as a result of vaccination. Challenge with infected ticks resulted in 20%, 57%, and 100% survival respectively.

2.7.4.2
Use of infected cells cultured in vitro

Following successful growth of parasitized lymphocytes in culture by Malmquist *et al*. (1970) attempts to immunize cattle were made and the results were summarized by Brown *et al*. (1970) as follows:

(a) Cattle could be infected by the subcutaneous or intravenous routes of inoculation with 10^7 cells.

(b) The macroschizont stage completed the life cycle in the bovine host, and intra-erythrocytic piroplasms were produced. The piroplasms were infective for R. appendiculatus ticks.

(c) The infected ticks when fed on susceptible cattle injected infective particles of T. parva which led to classical ECF.

(d) 10^9 cells caused a fatal ECF reaction.

(e) Between 10^5 and 10^9 cells led to mild or inapparent reaction and cattle after such exposure were resistant to tick-induced lethal infections.

(f) Immunogenesis depended on establishment of the parasite in cattle, and

(g) Infectivity and immunogenesis declined with maintenance and/or passage in vitro.

Later, Brown et al. (1978) stated that infectivity was quantum dependent and varied between cell lines with different T. parva strains and passage. To illustrate the point they summarised results on two cell lines with two strains of T. parva. Cell line C379 infected with T. parva (Aitong) at passage 15 killed 70% of cattle inoculated with 10^8 cells but did not kill any at passage 319 - and the survivors were immune to challenge with lethal stabilate material. On the other hand, cell line C2 with T. parva (Muguga) between passages 24-31 killed 12% of cattle inoculated with 10^8 cells but none between passages 49-205, and 36

out of 37 cattle were immune on challenge with homologous stabilate.

2.7.4.3

Attenuation of Theileria in infected-lymphoblasts in mice:

Other investigations with regard to use of parasitized lymphocytes were carried out by Irvin et al. (1976) who reported adaptation and possible attenuation of T. parva-invected bovine cells in irradiated mice. T. parva infected cells taken from cattle immediately after death, when inoculated into groups of irradiated Swiss and athymic nude mice, became established in one group of Swiss mice and in two groups of athymic nude mice. Cells from one of these isolates in athymic mice were passaged six times through mice. Cattle inoculated with the mouse passaged cells showed mild reactions and subsequently resisted a lethal ECF Challenge. They suggested that the possibility of vaccinating cattle against ECF by means of mouse passaged cells merited further studies.

2.7.4.5

Use of schizont stage for immunization against T. lawrencei

Although not much work has been carried out on use of T. lawrencei infected cells, their potential for immunization was shown by Young et al., (1973) during cross-immunity studies between T. lawrencei and T. parva. They found that cattle inoculated with in vitro grown cells containing

T. lawrencei schizonts survived a subsequent *T. lawrencei* challenge.

2.7.4.6

Use of infective particle stage

Two other methods of immunization have been investigated but they are only briefly mentioned in this thesis since they do not involve use of the schizont stage of the parasite which is the main subject of the thesis.

a) Quantum of infection method

This method was based on a hypothesis that there is a threshold of infective material below which parasites would not become established in cattle (Wilde *et al.*, 1968), and that around this threshold level a range of dosages would evoke a response varying from non-clinical establishment of the parasite to fatal disease. The hypothesis was tested by Cunningham *et al.* (1974) with the aim of determining a dose capable of evoking protective immunity and a non-clinical establishment of parasite concurrently. They were unable to obtain uniform reactions in cattle apparently given identical aliquots of infective particles, and concluded that this method of immunization would be unacceptable.

b) Chemoprophylaxis (infection and treatment)

The other method of immunization is chemoprophylaxis. It is based on observations by Neitz (1953) that cattle exposed to *Theileria* infection while they are concurrently being treated with tetracyclines develop protective immunity on recovery. However, tetracycline administration following

overt disease is ineffective. In other words the drugs have a chemoprophylactic effect but no chemotherapeutic effect.

The method was impractical in the form it was first used by Neitz (1953) because the drug had to be administered for more than a week to produce desired results. An FAO report (Anon.,1976) indicated that the method had been improved to a level where both the drug and infective particles could be inoculated on the same occasion and obtain protection of the animal against a homologous challenge. However, Purnell (1977) has suggested that a single administration of the drug is sometimes insufficient to suppress development of the parasite and could therefore lead to induction of a carrier status in the animal because piroplasms infective for ticks might occur.

2.7.5
T. annulata immunization

2.7.5.1
Use of blood and homogenates of lymphoid tissues:

Sergent et al. (1932) and Adler and Ellenbogen (1934) immunized cattle by inoculating blood from an animal in the acute stage of T. annulata infection with a relatively mildly virulent strain of the parasite. One of the mild strains isolated by Sergent et al. (1932) was the 'Kouba' strain, it caused 3% mortality in artificially infected cattle and had lost ability to produce intraerythrocytic piroplasms after 18 passages through cattle. Since the strain used for immunization was not always antigenically identical with the local strains cattle were reinoculated with a local strain to reinforce immunity (Tsur 1949).

In the U.S.S.R. there have been many attempts to experimentally immunize cattle with blood or blood with spleen suspensions (Mirzabekov *et al.*, 1969; D'Yakonov *et al.*, 1975; Askarov, 1975) but these attempts have not been followed by field application of the techniques.

2.7.5.2

Use of infected lymphoblasts cultured *in vitro*

One of the major disadvantages of using infected blood or homogenate tissues from spleen for immunization is the lack of standardisation of the vaccine because different donor animals would have to be used each time. These problems were partly overcome when Pipano and Tsur (1966) demonstrated that tissue culture material of *T. annulata* was infective for cattle and the virulence of such material decreased with prolonged maintenance *in vitro*. They found cattle inoculated with such material were immune on challenge with infected blood from animals at the acute stage of theileriosis. The cultured material was safer than infected blood since no clinical reactions followed inoculation of *in vitro* attenuated schizonts into all kinds of cattle, including pregnant dairy cows (Pipano *et al.*, 1973). However, Hashemi-Fesharki and Shad-Del (1973) detected parasites and febrile responses in cattle inoculated with cell culture vaccine although these occurred with less frequency than in animals inoculated with virulent organisms from blood. Pipano (1976) thought that the degree of virulence that occurred with cell culture material used by Hashemi-Fesharki and Shad-Del (1973) was due to incomplete attenuation.

Despite a few reactions live cell culture vaccine is now widely used in areas where T. annulata infections occur as evidenced by reports of Pipano (1976) in Israel, Stepanova et al. (1977) in the U.S.S.R., Gill et al. (1976) in India and Hashemi-Fesharki (1973) in Iran.

Pipano et al. (1977) reported on immunization of cattle against T. annulata using killed schizont vaccine. Cultured lymphoid cells with T. annulata schizonts were disrupted by freeze-drying and sonication. Calves were inoculated with fractions of the disrupted cells with and without Freund's complete adjuvant. Highest degree of protection against blood challenge was in calves receiving vaccine with the adjuvant, the particulate portion of disrupted parasites being more effective in protecting than the soluble. High IFA titres developed in all animals receiving vaccine with the adjuvant but no relationship was observed between antibody titre and protection against challenge. Calves receiving killed schizonts were immune to challenge with infected blood but remained completely susceptible to Theileria infective particles.

2.7.5.3

Chemoprophylaxis using the infective stage in the tick

Live cell culture vaccine has been apparently so successful that not many reports involving T. annulata chemoprophylaxis appears in the literature. However, Gill et al. (1976) successfully immunized cross-bred calves by administration of 16 mg kg^{-1} bodyweight chlortetracycline daily for 4, 8 and 16 days and simultaneous application of infected ticks.

On challenge recovered calves developed mild reactions with no mortalities while untreated control calves suffered 56-66% mortality.

2.7.6

T. mutans

This parasite is generally regarded as non-pathogenic and Neitz (1957) reported that no vaccine was available for immunization. He recommended that cattle in enzootic areas should be immunised against anaplasmosis, babesiosis and heartwater to avoid complications that arise due to concurrent T. mutans infections. However, since the realization that some strains of T. mutans were capable of causing fatal infection in T. parva susceptible and immune cattle, and that T. mutans could therefore serve as complicating factor in ECF immunization in East Africa, attempts were made to inoculate blood carrying apathogenic T. mutans strains into cattle being immunised against ECF. An FAO report (Anon., 1976) suggested that considerable benefit was conferred by the procedure. In a recent review on recent research on ECF in East Africa, Purnell (1977) suggested that since T. mutans tick derived stabilates can now be produced (Young et al., 1978) an immunogenic "cocktail" stabilate acceptable throughout the region might have to contain a T. mutans element.

2.7.8

Cross-immunity

The cross immunity between T. parva and T. lawrencei is good but not always sufficient to prevent clinical

reinfection and, in general, T. lawrencei seems to engender greater protection against T. parva than vice versa (Cunningham et al., 1974a, Young et al., 1973, Radley et al., 1975b). There is no immunity to reinfection between any other theilerial species.

There are, however, Theileria strains or species in the field to which T. parva or T. lawrencei show no cross-immunity. Burridge et al. (1974) reported such a parasite. They isolated a theilerial parasite infective for cattle from R. appendiculatus at Githunguri, Kiambu district, Kenya. 8 cattle, experimentally infected, developed transient fever and small numbers of schizonts and intra-erythrocytic parasites. All cattle recovered but were fully susceptible to a T. parva (Muguga) challenge. IFA test studies revealed that the Githunguri parasite was antigenically distinct from T. parva, T. lawrencei and T. mutans although it had some common antigens with T. parva and T. lawrencei. A similar lack of cross protection was reported by Sergent et al. (1945) between Algerian and Israeli strains of T. annulata.

2.8 Theileria cell culture

Of all the stages of development of Theileria, only the lymphocytic stage can be grown in vitro to date. This can be achieved in two ways: either by establishing infected cells in culture from infected bovine lymphoid tissues or by incubating the infective particles harvested from the tick with uninfected clean bovine lymphoid cultures. In subsequent

pages, the development of Theileria cell culture and studies carried out, so far, on these in vitro maintained cells will be discussed.

2.8.1 History of Theileria cell culture

2.8.1.1 Earlier attempts to grow Theileria spp. in culture

Cultivation of T. annulata was first reported by Tsur (1945) and further details were given in a subsequent publication (1953). In tissue, incubated in serum and Tyrode solution at 38° C, macroschizonts survived for 12 days but did not multiply. Fresh fragments of spleen added to the cultures extended survival to between 15 and 18 days but no multiplication occurred. Addition of glutamine, pyridoxine, riboflavine and inositol together to the medium led to multiplication. Growth was unaffected by use of immune serum or presence of penicillin and streptomycin in the medium. Tsur, later, thought that inositol was not essential for growth despite his earlier beliefs.

Brocklesby and Hawking (1958) confirmed the findings of Tsur (1945, 1953) but did not consider addition of glutamine, riboflavine and pyridoxine essential for growth. In the same publication, they noted that macroschizonts could only be found in lymphoid cells and possibly macrophages but none in fibroblasts, an observation later confirmed by Tsur and Adler (1963). Unlike T. annulata, T. parva grew less readily, multiplication taking place in the first week and schizonts survived up to 15 days (Tsur et al., 1957;

Brocklesby and Hawking, 1958). Addition of growth factors did not improve growth prospects of T. parva and no macroschizonts broke up into merozoites during in vitro cultivation.

2.8.1.2
Mass cultivation of Theileria spp.

Tsur and Adler (1963) for the first time grew T. annulata on a large scale by monolayer tissue culture method. This was done by re-suspending trypsinized infected bovine cells in a medium of Earle's salts solution supplemented with serum, yeast and antibiotics. Theileria-infected cells were maintained and multiplied in culture for more than 76 days. Later, Hulliger (1965) carried out mass cultivation of T. annulata and T. parva for over 7 months and T. lawrencei for over 3 months, in association with baby hamster kidney (BHK) cells. She used a medium containing 10% calf serum, 10% tryptose phosphate broth (2% stock solution) and 80% Eagle's medium modified according to Stoker and Macpherson (1962). She was of the opinion that BHK cells served as a feeder layer and were probably best suited for this role because of their rapid growth rate leading to favourable metabolic conditions during early cultivation and also because of their whorl-like fibrocytic pattern which trapped bovine lymphoid cells on the bottom of the flask where multiplication was favoured. She suggested that the monolayer technique of Tsur and Adler (1963) probably had two cell types and the fibrocytic type served the same role as the BHK cells in her experiments.

Brocklesby and Hawking, 1958). Addition of growth factors did not improve growth prospects of T. parva and no macroschizonts broke up into merozoites during in vitro cultivation.

2.8.1.2
Mass cultivation of Theileria spp.

Tsur and Adler (1963) for the first time grew T. annulata on a large scale by monolayer tissue culture method. This was done by re-suspending trypsinized infected bovine cells in a medium of Earle's salts solution supplemented with serum, yeast and antibiotics. Theileria-infected cells were maintained and multiplied in culture for more than 76 days. Later, Hulliger (1965) carried out mass cultivation of T. annulata and T. parva for over 7 months and T. lawrencei for over 3 months, in association with baby hamster kidney (BHK) cells. She used a medium containing 10% calf serum, 10% tryptose phosphate broth (2% stock solution) and 80% Eagle's medium modified according to Stoker and Macpherson (1962). She was of the opinion that BHK cells served as a feeder layer and were probably best suited for this role because of their rapid growth rate leading to favourable metabolic conditions during early cultivation and also because of their whorl-like fibrocytic pattern which trapped bovine lymphoid cells on the bottom of the flask where multiplication was favoured. She suggested that the monolayer technique of Tsur and Adler (1963) probably had two cell types and the fibrocytic type served the same role as the BHK cells in her experiments.

Although the work of Tsur and Adler (1963) and Hulliger (1966) seemed to imply that monolayers were indispensible for cultivation of Theileria spp., Malmquist et al. (1970) demonstrated that it was possible to subculture Theileria-infected lymphoblasts readily without feeder layers. The latter workers used Eagle's Minimum Essential medium supplemented with 20% foetal calf serum, $0.10g.l^{-1}$ of L-asparagine and antibiotics as growth medium. They, however, observed that plating of infected lymphoblasts was greatly enhanced by presence of preformed splenic monolayers in culture flasks. The significance of preformed bovine embryonic spleen monolayers was further confirmed by Stagg et al. (1974) who established Theileria-infected buffalo cell lines from blood in which no macroschizonts had been detected on microscopic examination prior to setting up cell cultures. All cultures set up without monolayers failed to give rise to Theileria-infected buffalo cell lines while all cultures set up on monolayers gave rise to Theileria-infected buffalo lymphoblastoid cell lines that could eventually be subcultured without monolayers.

2.8.1.3
Establishment of Theileria-infected cell lines from infective particles and uninfected cattle cells

It is possible to establish Theileria-infected cell lines by incubation of infective particles from the tick with uninfected bovine lymphoid cells in culture (Brown et al., 1973). The infective particles used are either harvested by the in vitro feeding technique of Purnell and Joyner (1968),

or by grinding infected pre-fed nymphs or adult ticks in Eagle's Minimum Essential Medium supplemented with 3.4% bovine serum albumin (Fraction V from bovine plasma) and subsequently collecting the supernatant (Cunningham *et al.*, 1973a). This technique of *in vitro* infection has potential for the *in vitro* titration of infective particles (Brown *et al.*, 1973). This technique has potentially many other advantages. For instance such *in vitro* titration would cut dramatically the costs of research on theileriosis by avoiding use of whole animals and it would also provide an opportunity for studying the life cycle of the parasite with regard to the occult phase of the parasite (Radley *et al.*, 1974) which comes prior to the uninucleate lymphocytic stage.

That, briefly outlines the development of *Theileria* cell culture. Improvement of the techniques for propagation of these cells in culture has provided opportunities for certain studies to be carried out on the infected lymphoblast. In subsequent chapters studies carried out by various workers, which have a bearing on this thesis will be discussed, nevertheless transformation of lymphoid cells by the parasite will be discussed in this review so as to make it easier to follow work of this thesis.

2.8.2

Transformation of reticulum cells to lymphoblasts by T. parva

The term transformation was first introduced to *Theileria* cell culture by Malmquist *et al.* (1970). They used the term to describe spleen cell lines parasitized by

T. parva because of the unique properties of these cells namely, being refractile, growing in suspension continuously in vitro and showing marked motility, phenomena hitherto unobserved in cultured normal bovine spleen cells. They suggested that transformation was initiated by the parasite which lead to an indeterminate number of replicative cycles in vitro.

Although the term had been used, Moulton et al. (1971) were not sure whether the process was transformation or transplantation. As a result, they carried out a series of experiments to investigate the phenomenon. They set up spleen cultures from 6 infected cattle and three non-infected animals (one from a calf and two from bovine foetuses) using the methods of Malmquist et al. (1970). Using light and electron microscopy they observed the attachment of macrophages on to the surface of culture flasks followed by transformation of macrophages into reticulum cells which formed a monolayer throughout the flask. Reticulum cells from cultures of infected spleens developed into infected lymphoblasts while reticulum cells in cultures of non-infected spleens developed into lymphocytes. They interpreted this as evidence for transformation of reticulum cells by the parasite. To verify the above view, a carbon particle uptake study was undertaken with the aim of tracing the origin of infected lymphoblasts in suspension. Results obtained showed presence of carbon particles in a lymphoblast population originating from monolayers previously washed to remove initial lymphoblasts in suspension prior to addition of carbon particles. On the other hand, there were no carbon particles in infected lymphoblasts that had been previously

passaged into new flasks to which carbon particles had been added. These findings indicated that non-phagocytic lymphoblasts contained carbon particles when they developed from transforming reticulum cells. On the basis of the above observations Moulton, et al. (1971) concluded that the process was transformation and not transplantation.

Malmquist and Brown (personal communication) do not necessarily accept the view by Moulton et al. (1971) and believe that infected lymphocytes become trapped in the monolayer and their daughter cells come off in the supernate. They maintain that careful examination of a pre-transformed culture will always show infected lymphoid cells. Moulton et al. (1971) have never showed an infected reticulum cell.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1
Experimental animals

3.1.1
Buffaloes:

They were African Cape buffaloes (Syncerus caffer caffer). Seven of these buffaloes had Theileria piroplasms in their blood while the eighth did not. Table V summarizes information on site of capture, place of captivity and sex of the animal.

3.1.2
Cattle

3.1.2.1
Non-infected cattle

Information on uninfected cattle used to obtain lymphoid cells is as detailed in Table VI.

3.1.2.2
Theileria-infected cattle

Experimentally Theileria-infected cattle were initially obtained from farms exercising strict tick control programmes in Kenya. They were shown not to have antibodies to T. parva by the IFA test (Burridge and Kimber, 1972). These animals were therefore presumed susceptible to ECF. They were then inoculated with either T. lawrencei or T. parva

stabilates of infective particles (VII) for purposes of immunochemoprophylaxis (Radley et al., 1975b) or stabilate production (Cunningham et al., 1973a, b). The immunized cattle (Table VIII) were later exposed in a buffalo paddock for challenge purposes. Table VIII summarizes the history of cattle immunized and exposed to a T. lawrencei challenge in the buffalo paddock, and one steer inoculated with a suspension of infective particles for the preparation of stabilate.

3.1.2.2.1

Buffalo paddock (Young et al., 1977a)

This is a paddock at the East African Veterinary Research Organization (EAVRO), Muguga, set up for purposes of evaluating methods of immunization against T. lawrencei infection (Young et al., 1978b). Originally there was a Theileria-free tick population in the paddock, then two T. lawrencei carrier buffaloes were introduced. These led to infection and maintenance of T. lawrencei in ticks. Subsequently, successive groups of cattle were introduced to monitor T. lawrencei transmission. Lethal infection in cattle was first observed 10 months after introduction of the buffaloes. Engorged uninfected female R. appendiculatus were then introduced to boost the tick population in the paddock. Lethal T. lawrencei infections in non-immunized, and in some cases immunized cattle has continued ever since.

3.2
Collection of biopsy and autopsy specimens

3.2.1
Spleens

Small pieces of spleen from either foetuses or adult cattle dying of Theileria infection were aseptically collected into sterile jars containing medium F 12 (see Media). In the laboratory cells were teased from the pulp in a petri dish containing F 12. The spleen capsule was discarded and clumps of cells broken down by pipetting. The resultant cell suspension was centrifuged at 160 x g for 10 minutes, the supernatant discarded. The fibrocytic cells together with mononuclear cells were then carefully removed with a pipette taking care to avoid excessive contamination with erythrocytes below. The cells removed were then resuspended into growth medium (see Media). No attempts were made to estimate number of cells harvested but a small aliquot was withdrawn for making smears to be stained with Giemsa stain and examined for presence of parasites.

3.2.2
Lymph nodes

3.2.2.1
From cattle infected with Theileria (Malmquist and Brown,1974)

Lymph node samples from carcases were processed in a similar way to that described for spleens above. However, lymph node biopsies were obtained from a shaven and washed area which was then disinfected with 70% alcohol. A 10 ml

syringe containing 5 ml of medium F 12 and fitted with a 39mm needle with bore diameter of 1.52mm was used to probe the node while gradually pulling the plunger to suck cells and fluids from the node. The contents were expelled into a universal bottle containing 5 ml of medium F 12. The suspension was later centrifuged at 160 x g for 5 minutes and the supernatant discarded. Smears for Giemsa staining made from the deposit which was then resuspended in growth medium.

3.2.2.2
From non-infected cattle

The method used was based on the one described by Peters (1975). Immediately following slaughter, the prescapular lymph nodes were dissected out, and transferred to a jar with medium F 12. In the laboratory, the node was freed from the surrounding tissue, the capsule incised and cells teased from the parenchyma into medium F 12 in a petri dish. The suspension was centrifuged at 160 x g for 3 minutes to deposit large cell clumps which were discarded. The supernatant was then centrifuged at 400 x g for 10 minutes. The pellet was then resuspended in 3 ml of isotonic PBS pH 6.8 and layered on lymphoprep (Appendix 1). When centrifuged at 400 x g for 5 minutes, erythrocytes were sedimented to the bottom of the tube while mononuclear lymphoid cells remained at the interface. The mononuclear cells were then washed once in isotonic PBS and then resuspended into medium F 12. 0.5 ml of the suspension was used to estimate viability of cells and to make smears for staining with Giemsa.

3.2.3

Blood

Venous blood was collected from both infected and non-infected buffalo and cattle. Buffaloes were tranquillised with Rompun before attempts were made to collect blood. The area over the jugular vein was disinfected with 70% alcohol and blood withdrawn by puncturing the vein with a 14- or 16-gauge siliconized needle allowing blood to run freely into a bottle containing 5000 units of heparin sodium solution. 100 ml of blood were collected resulting in 50 units of heparin per 1 ml of blood. Below is the method of leukocyte separation, based on an FAO report (1978).

3 ml of the blood were used to make smears and to estimate leukocyte counts per ml. The remaining blood was chilled at 4° C for 10 minutes, then centrifuged at 2000x g for 20 minutes at 4° C, and plasma discarded leaving about 15 ml over the layer of buffy coat. The buffy coat was removed with a 2 ml wide mouthed-pipette, mixed with isotonic PBS at pH 6.8 and layered on 3 ml of lymphoprep in a round bottomed siliconized 10 ml centrifuge tube. Separation of leucocytes and platelets from erythrocytes occurred when the suspension was centrifuged at 400 g for 25 minutes at room temperature but if three layers were not apparent, then separation was incomplete and centrifugation was continued for a further 15 minutes. The topmost cell free layer was discarded while the interface, containing leukocytes and platelets, was carefully removed, avoiding contamination with erythrocytes in the lower layer. The leukocyte-platelet suspension was transferred to a centrifuge tube containing 8 ml of isotonic PBS then centrifuged for 10 minutes at 160xg and supernatant

discarded. The pellet was resuspended in isotonic PBS and centrifuged as before. This reduced the number of platelets with the harvested mononuclear cells. The resulting cell pellet was resuspended in growth medium and sample withdrawn for estimation of cell numbers, cell viability and smears for Giemsa staining.

3.3 Cell culture

3.3.1 Flasks and media

Cultures of tissues from experimental animals were set up and maintained in 25 ml plastic culture flasks (Falcon Plastics) as static cell cultures. 250 ml plastic flasks were used for pooling cells.

Three types of culture media were employed in the course of this work and were adjusted to pH 7.2 during preparation (see Appendix 2). They were all produced by Grand Island Biological Company (GIBCO). Medium F 12 is Eagle's minimum essential medium (MEM) with Hank's salts base. This medium was used for collection of lymph node and spleen samples from animals. 10 units of heparin sodium per ml were added to F 12 just before use. The other two media were F 11 (Eagle's MEM with Earle's salts base) and RPMI 1640. RPMI 1640 is suitable for growth of both monolayer and suspension cells while F 11 is mainly suitable for monolayer cultures.

All 3 media received supplements as follows: 0.100 gm. l^{-1} of L-β-asparagine, 100 international units (i.u.) ml^{-1} of penicillin, 100 ug ml^{-1} of streptomycin and 100 ug ml^{-1} of Kanamycin. F 11 and RPMI 1640 were further supplemented with 20% foetal calf serum (FCS) before use as growth media. However, in this thesis, growth media refers to F 11 with 20% FCS unless otherwise specified that growth media was RPMI 1640.

3.3.2
Setting up and maintenance of cultures

Cells harvested as described earlier in this chapter, were used to set up either fibroblastic feeder layers from embryonic spleens, or Theileria-infected cells in culture from infected animals or uninfected lymphoid cell cultures for mitogen stimulation.

3.3.2.1
Bovine embryonic spleen cells (BESP) (also called feeder layers) (Malmquist et al., 1969)

Cells harvested from bovine foetal spleens (Table VI) were introduced into 25 ml culture flasks, gassed with 5% carbon dioxide (CO_2) - 95% air mixture and incubated at 37° C. At intervals of 3 days old medium and unattached cells were replaced by fresh growth medium. The monolayer usually completely covered the flask surface within a week; at this stage the serum supplement in the growth medium was reduced to 10% (v/v). The medium was replaced once a week.

When cells became overcrowded the monolayers were treated with a trypsin solution (Appendix 3). Old medium was discarded, the culture flask washed with trypsin solution and then 5 ml of the solution introduced and incubated at 37° C for 10 minutes. 5 ml of growth medium were then added to the flask and the resulting 10 ml cell suspension centrifuged at 400 "g" for 5 minutes. The old flask was washed twice with growth medium which was discarded. Then the cell pellet was resuspended in growth medium, dispensed into the old washed culture flask and two new culture flasks. Flasks were gassed with 5% CO_2 air-mixture and incubated at 37° C as before.

3.3.2.2

Theileria-infected cell cultures from spleens of infected cattle (Malmquist et al., 1970)

Cultures were set up in exactly the same way as described above for the feeder layers. However, the cells in old medium were sedimented by centrifugation then resuspended into fresh growth medium and reintroduced into the flask whenever medium was being changed. This was done at intervals of 3 to 4 days until either transformation (see Chapter 2) occurred or the cultures were discarded.

Whenever a healthy lymphoblastoid cell population became apparent, 2 ml of cell suspension were removed for making smears to be stained with Giemsa for parasite examination. On the same occasion the sample taken was replaced by 2 ml of fresh growth medium. This procedure did not affect

the 3 to 4 day medium changes as the smears were made on a different occasion.

When density of the lymphoblastoid cell population became too high for the original culture some of the cells were subcultured into a new flask. The flask was gently shaken to detach some of the monolayer cells. The cell suspension was centrifuged and the pellet resuspended into 6ml of fresh growth medium. 1 ml of the cell suspension was reintroduced into the old flask together with 9 ml of fresh medium while 5 ml were introduced into a new culture flask, gassed with 5% CO_2 air mixture. Both cultures were incubated at 37° C. After two to three subcultures infected lymphoblastoid cells could be subcultured successfully into new flasks without the concomitant fibroblastic cells provided that 10^5 or more infected cells per ml were used.

3.3.2.3

Theileria-infected cell cultures from lymph nodes and blood of infected animals

Cells of these tissues (Tables XI and XII) were either introduced into culture flasks with preformed bovine embryonic spleen feeder layers or set up into new culture flasks without feeder layers. Both types of cultures were handled in exactly the same manner described for cultures from infected spleens.

3.3.2.4

Uninfected lymphoid cell cultures for mitogen stimulation

Cultures of these cells (Table VI) were set up as described for the infected spleen cultures, however, none of

the cultures were maintained for more than 4 days since they were only used for mitogen stimulation.

Mitogen stimulation:

Two mitogens were used, Concanavalin A (Con A) (Pharmacia) a T-lymphocyte mitogen and Lipopolysaccharide B (LPS) extracts of Escherichia coli (DIFCO), a B-lymphocyte mitogen. Doses used were those found to give optimal stimulation of bovine lymphocytes by Rouse and Babuik (1974).

Two types of cultures were set up, in RPMI 1640 with 10% FCS, for each cell batch harvested, namely, 1 ml and 10 ml cultures. 1 ml cultures were in 1 ml glass screw-capped ampoules while 10 ml cultures were in 25 ml Falcon plastics flasks. They were set up as shown below:

Mini cultures	Macro cultures
3 x 1 ml cultures without mitogen	1 x 100 ml culture without mitogen
3 x 1 ml cultures with Con A	1 x 100 ml culture with Con A
3 x 1 ml cultures with LPS	1 x 100 ml culture with LPS

Mini and macro cultures with 10^6 lymphoid cells per ml received mitogens at the rate of 6.25 ug of Con A per ml and 25 ug of LPS per ml. All cultures were then gassed with 5% CO_2 air-mixture and incubated at 37° C for 3 days.

On the third day mini cultures were pulsed with 1 uCi of (methyl-3H) tritiated thymidine of specific activity 5 ci/mMol. (The Radiochemical centre, Amersham). 24 hours later mini cultures were processed for estimation of amount

of tritiated thymidine incorporated in DNA of the cells while macrocultures were used to prepare enzyme extracts for isoenzyme studies.

Mini culture tubes were centrifuged at 400x g for 10 minutes, then supernatant discarded and the mouth of tubes blotted dry. Pellets were washed in 0.85% saline once, then in ice cold 5% trichloroacetic acid (TCA) twice and finally in methanol. The pellets were dissolved in 0.1 ml of 1 N sodium hydroxide for 60 minutes in a water bath at 56° C. 1 ml of methanol was then added to each culture tube and transferred to scintillation counting vials with 10 ml of liquid scintillation fluid (Emulsified Dioxane 299. Packard). Samples were counted in a Packard Tricarb III liquid scintillation spectrophotometer. The mean of counts from triplicate samples was used to estimate stimulation index. Stimulation index (S.I.) was obtained by dividing the average counts per minute of mitogen stimulated samples by the average counts of unstimulated cultures.

3.4

Pooling cells

The purpose was to build up a big number of cells which would be used for either cryopreservation or preparation of enzyme extracts (see below). 25 ml of 2-day old, actively growing Theileria-infected cells in growth medium were introduced into 250 ml Falcon Plastics culture flasks, and gassed and incubated as described previously. Two further volumes of growth medium (25 ml and 50 ml respectively) were added

at 2-day intervals and cells were harvested on the eighth day by centrifugation at 500 g for 10 minutes at 4° C.

3.5
Cells used in various experiments

These were Theileria-infected lymphoblastoid cell lines (Table IX), lymphoblastoid cell lines not infected with Theileria (Table X) and freshly harvested cells from lymphoid tissues (Table VI).

3.6
Cryopreservation

Only Theileria-infected lymphoblasts from cultures were cryopreserved. An FAO technical report procedure (Anon. 1978) was used.

3.6.1
Preparation of cooling containers and medium

Sterile 1 ml glass ampoules were labelled with the appropriate cell culture code and passage number and held in a small beaker on an ice tray. The canes were also labelled and kept in a cardboard tube at -20° C before use.

To 16 ml of fresh sodium bicarbonate free MEM without antibiotics 4 ml of FCS were added. This constituted solution A. To 8 ml of solution A 2 ml of sterile dimethyl sulfoxide (DMSO) were added and this constituted solution B. Both solutions A and B were held at -20° C prior to use.

3.6.2

Preparation of cells.

Cells were harvested as described in the section on bulking. After discarding the supernatant the cell pellet was resuspended in 5 ml of solution A, mixed thoroughly and 0.1 ml of cell suspension withdrawn for cell counts and viability estimation. 5 ml of solution B were added to the resuspended cells in solution A and the solutions thoroughly mixed. 1 ml cell suspensions were then dispensed into ampoules which were sealed in an oxygen-boosted gas flame, returning the ampoule on ice immediately after sealing. Subsequently ampoules were transferred to canes in the cardboard tube which was then transferred to a -70° C refrigerator (REVCO). 24 hours later the canes were transferred to canisters in a liquid nitrogen refrigerator noting in a record book the code number for the cell line, the number of ampoules stored and position of canisters in the refrigerator.

3.6.3

Checking viability of cryopreserved cells

This was done 24 hours after the transfer of canes into the liquid nitrogen refrigerator. Briefly, the procedure was as follows: holding the cane as low as possible in the refrigerator the topmost ampoule was removed and the cane then replaced in the refrigerator. The ampoule was subsequently transferred to a 37°C waterbath where the contents were rapidly thawed. The ampoule was then swabbed with absolute alcohol, the neck broken, flamed and the contents transferred

to 10 ml of prewarmed growth medium in a centrifuge tube. Cells were sedimented at 160 g for 5 minutes, supernatant discarded and cell pellet resuspended into fresh growth medium followed by gassing with 5% CO_2 in air and incubation at 37° C. The medium was replaced with fresh growth medium 24 hours later and thereafter medium was changed every 3 to 4 days.

3.6.4

Storage and transportation of cryopreserved cells

Following confirmation of viability of cryopreserved cells, the material was permanently kept in liquid nitrogen refrigerator throughout the period the material was in East Africa; during transportation by air to the UK and throughout the course of stay in the UK. During this period the liquid nitrogen refrigerator was always "topped up" once every week.

3.7

Chromosome preparations

Karyotypes of Theileria-infected cell lines isolated by the author were prepared. Methods used were based on those described in FAO Technical Report 3. (1978). All glassware except slides were siliconized.

0.20 ml of demecolcine (Malmquist et al., 1970)solution from a 10^{-5}gm. ml^{-1} stock solution were added to 10 ml of an actively growing 24-hour old cultures to give a final concentration of 2×10^{-7} gm. ml^{-1}. Cultures were incubated for 2 hours and the cells pelleted by spinning at 400 g for 5 minutes. Cells were resuspended in 5 ml of freshly prepared

hypotonic 0.5% sodium citrate solution and held at 37° C in a water bath for 35 minutes. Sodium citrate solution lysed the cells and helped disperse the chromosomes. The suspension was centrifuged at 400 "g" for 5 minutes and the pellets resuspended into 5 ml of ice cold acetic alcohol (1 volume of glacial acetic acid to 3 volumes of methanol), held at 4° C for 10 minutes and the pellet resuspended again in a fresh 5 ml of acetic alcohol. After another 10 minutes at 4° C the tube was then centrifuged and the pellet was resuspended into a volume of acetic alcohol equal to that of the pellet. Chromosome preparations were made by letting a drop of the suspension fall on to a very clean ice cold, wet slide from a distance of one foot. The slide was then dried above a Bunsen burner flame. The preparations were stained with 5% Giemsa stain at pH 7.2 for 40 minutes. A minimum of 50 karyotypes were examined for each cell line.

3.8 Microscopy

3.8.1 Light microscopy

3.8.1.1 Cultural features

These were examined using an inverted light microscope. Culture flasks were examined daily during attempts to establish Theileria-infected cells in culture otherwise examinations of established cell lines were made 12-24 hours after setting up culture and on the third or fourth day before change of medium.

3.8.1.2
Estimations of number of viable cells

Estimations of percentages of cells viable was carried out on cell samples harvested from *in vivo* and *in vitro*. An exclusion dye test using nigrosin stain to detect dead cells was used (Kaltenbach *et al*., 1958). A preparation was made containing equal parts of culture and 0.45% Nigrosin in PBS pH 7.2. After mixing gently and allowing the stain to act for 30 seconds a drop of cell suspension was introduced into a haemocytometer and estimation of both stained and unstained cells made from counts on both chambers of the haemocytometer.

3.8.1.3
Preparation and observations on Giemsa stained smears

Smears were made by removing 0.5 ml of culture cell suspension and centrifuging at 160xg for 5 minutes. Similarly 0.5 ml of cell suspensions freshly prepared from tissues of live or dead animals was used for smear preparations. Most of the supernatant was discarded and cell deposit resuspended in remaining fluid. A small drop of the cell deposit was transferred to the end of a clean glass slide. Another glass slide was brought into contact with the drop and the inclined slide pushed to the opposite end of the slide on to which the drop was placed.

Smears were air-dried, fixed in methanol and stained with 5% Giemsa stain at pH 7.2 for 40 minutes. Stained slides were then washed in running water for between 1 and 2 minutes

After air drying, the following observations were made on the smears:

(i) percentage of cells containing parasites

(ii) percentage of cells in mitosis

(iii) percentage of multinucleate cells

(iv) mean schizont nuclear number (MSN)

A total of five hundred cells were counted to obtain values for (i), (ii) and (iii). MSN was determined by counting Theileria nuclei in 50 intracellular schizonts and dividing the total count by 50.

3.8.2
Electron microscopy:

3.8.2.1
Preparation of specimens for scanning electron microscopy (SEM)

3.8.2.1.1
Harvesting, fixation, and dehydration of cells

Suspension of cells from 2-day old Theileria-infected cells or freshly harvested peripheral bovine leukocytes were centrifuged at 160xg for 5 minutes, then washed three times in RPMI 1640 serum-free medium and finally resuspended to give 10^5 cells per ml. 1 ml of resuspended cells was transferred to a filter holder containing 0.6 um pore size, nucleporemembrane. The cells were deposited on the membrane by flushing 3% glutaldehyde in 0.066 M cacodylate buffer (pH 7.4) through the filter holder three times. The filter holder was then filled with the fixative and immersed in a beaker of fixative for 24 hours at 4° C. The cells were

then rinsed twice with cacodylate buffer and dehydrated in the following graded series of acetone and water mixtures, changing solutions every 5 minutes: 30%, 30%, 50%, 70%, 80%, 90%, 100% and 100%.

3.8.2.1.2
Critical point drying

The aim of critical point drying is to dry a specimen without distorting its shape through surface tension effects of evaporation by the fluid in which it is immersed.

Specimens were transferred in absolute acetone to a precooled (20° C) high pressure chamber (Polaron E3000) of a critical point drying apparatus. The chamber was flushed with liquid carbon dioxide until all acetone was removed. Then carbon dioxide was allowed to soak through the specimen for 30 minutes twice so as to get rid of all traces of acetone from the cells. The temperature of the chamber was then raised to 40° C and at a pressure of 1200 lb/in^2 beyond the critical point for CO_2 (which is 32° C at a pressure of 1200 lb/in^2). At this point, CO_2 was slowly and carefully vented for 3 to 5 minutes, in order to avoid condensation effects due to rapid cooling.

3.8.2.1.3
Examination

The critically dried specimens were mounted on specimen stubs and coated with a thin film of gold approximately 50 nm thick to avoid charging under the electron beam.

To do this, an Edward's splatter coating unit was used. Coated specimens were then examined.

3.8.2.2

Transmission electron microscopy

3.8.2.2.1

Harvesting, fixation, dehydration and embedding of specimens

Cell suspensions from 2-day old cultures were treated by a modified Kellenberger et al. (1958) process used for fixing and embedding bacteria. First the cells were sedimented at 400xg for 5 minutes; the supernatant was discarded and 3% glutaldehyde in 0.066 M cacodylate buffer added to the pellet which was then kept at room temperature for 60 minutes. The fixative mixture was decanted and fresh cacodylate buffer added and kept at room temperature for another 60 minutes after which the pellet was infiltrated with 2% Agar to prevent it breaking up while being postfixed in 1% osmium tetroxide in cacodylate buffer for 30 minutes again at room temperature. Post fixed samples were washed in cacodylate buffer, initially for 2 hours and then overnight, then dehydrated in a graded series of methanol water mixture as follows:

% methanol:	30	30 with /acetate	60	70	80	90	100
Period of dehydration: (min.)	10	30	10	10	10	10	10
Number of times:	2	1	1	1	1	1	2

Next, specimens were treated twice for 10 minutes each in propylene oxide, an intermediate solvent used to facilitate penetration of the araldite resin. To ensure

thorough infiltration of the araldite specimens were then left in araldite for 30 minutes before changing into a fresh aliquot of araldite and leaving overnight. The next day specimens were again transferred into fresh araldite in which polymerization was carried out over a period of 48 hours.

3.8.2.2.2

Cutting and examination

Sections were cut using glass knives on an OMU2 model Reichert microtome. Initially sections about 1 µm thick were taken from each block, these were stained with 1% Toluidene Blue in Borax and examined by light microscopy; only blocks which showed numerous parasites were processed further.

For EM examination, sections 25-30 nm thick were collected on Smithurst New 200 grids without supporting films and then stained with uranyl acetate followed by lead citrate (Venable and Coggeshell, 1965). Specimens were examined with an AEl 801 electron microscope using an accelerating voltage of 80 KV and photographed on an Ilford SP353 film.

3.9

Isoenzyme investigations

3.9.1

Preparation and storage of lysates from infected and non-infected cells

Cells harvested from cell cultures and non-infected animal lymphoid tissues as described earlier were transferred to 8 ml plastic centrifuge tubes and washed in PBS (pH 7.2)

three times at 500 "g" for 5 minutes. 0.2 ml cell suspensions were withdrawn prior to the third washing for making Giemsa stained smears and to estimate cell viability and numbers. Following the third washing, the supernatant was discarded and excess fluid blotted from the packed cells. The cells were lysed by mixing with an equal volume of a hypotonic solution of enzyme stabilizers (1 mM EDTA, 1 mM dithriothreitol and 1 mM e-amino caproic acid) (Godfrey and Kilgour, 1976), then kept on ice for 10 minutes and frozen at -20° C for about 20 hours. After thawing at room temperature the lysed cells were spun at 3800 "g" for 60 minutes in a precooled (4° C) centrifuge. The supernatant now referred to as lysate was stored as 20 µl beads in liquid nitrogen. Beads were made by dropping 20 µl portions of supernatant into liquid nitrogen and stored in precooled, labelled, screw-capped, 1 ml glass ampoules with holes in the lid. They were held inside canes and canisters of a liquid nitrogen refrigerator.

3.9.2
Estimation of protein content of lysates

Protein estimation was carried out using a modified Lowry method (Kilgour, personal communication).

Reagents:

The following reagents were employed in the test:

2% $Na_2 CO_3$ in 1% Na K Tartrate	= Solution A
0.5% $CuSO_4 .5H_2O$ (in water)	= Solution B
50 ml of solution A + 1 ml of solution B	= Solution C
Folin and Ciocalteau's reagent (I.0 N)	= Solution D
IM NaOH	
5% Bovine serum albumin (BSA) (Sigma)	

Preparation of standard and test solutions

Standard solutions of 50, 100, 200 and 300 mg of protein per ml were prepared by diluting 5% Bovine serum albumin with water. Thawed enzyme extracts were diluted in distilled water to give 1:50, 1:100 and 1:200 dilutions; these were the test samples whose protein content was to be determined.

The method

Sodium hydroxide, solutions C and D were added in the sequence shown below to each standard solution, diluted test sample and control:

BSA standards:	Control:	Test samples:	
0.5 ml standard	0.5 ml H_2O	100 µl sample	Mixed and left to clear at room temperature
0.5 ml 1M NaOH	0.5 ml 1M NaOH	100 µl 1M NaOH	
5.0 ml solution C	5.0 ml solution C	1000 µl solution C	Mixed and left for 10 minutes
Add 0.5 ml solution D	0.5 ml solution D	100 µl of solution D	Mixed immediately tube by tube and left standing for 40 minutes

Absorbance units for each standard and test sample in cuvettes were read on a spectrophotometer initially adjusted to zero with the control solutions at 660 nm.

To obtain a standard curve, absorbance units of standard solutions were plotted against the respective milligrams of protein on a graph. Milligrams of protein, corresponding to absorbance units from different dilutions, were read off the curve and the protein was calculated from the mean of two or more readings within the standard curve.

3.9.3
Estimation of enzyme activities in lysates

In this exercise, units of enzyme activity of samples of lysates were determined for two enzymes (glucose phosphate isomerase and aldolase). A unit (U) of any enzyme is that amount of enzyme which will catalyse the transformation of 1 micromole (1 µmole) of a substrate per minute under defined conditions of temperature, pH and substrate concentration (Dawson et al., 1974).

5 µl of thawed lysate was added to 95 µl of 5% BSA in a small plastic tube and mixed thoroughly. 10 µl of diluted lysate was added to 1000 µl of a reaction mixture (Appendix 4 and 5) in each 1 ml cuvette.

Cuvettes containing control samples without substrate and test samples in duplicate were read on a Pye Unicam Spectrophotometer at 340 nm and absorbance units plotted at one minute intervals by a recorder connected to the Spectrophotometer.

Absorbance units were calculated from a regular reaction change recorded as a straight line through points (Fig. 2) as early as possible over a period of 5 minutes.

Absorbance units were then converted to enzyme units by the following formula:

$$\frac{Au}{T} \times \frac{CV}{L} \times \frac{OLD}{} \times \frac{1}{k}$$

where, Au are the absorbance units,

T, the time,

CV, the total volume of solution in cuvette,

OLD is the original dilution of lysate,

L is the volume of diluted lysate added to the reaction mixture, and

k is the coefficient of extinction and equals $\frac{1}{6.22}$ for NAD(H) and NADP(H).

3.9.4

Preparation of electrophoresis buffers

The appropriate buffer (Appendix 6) was poured into the anodic and cathodic compartments of the electrophoretic tank and the level made even (Fig.3).

Gel buffers were prepared by diluting appropriate tank buffers in the ratios shown in Appendix 6.

3.9.5

Preparation of thin layer starch-gel for electrophoresis

A starch gel 1 mm thick was prepared on glass plates with a 1 mm deep plastic rim (Fig. 4a). Each plate required approximately 40 ml of starch solution made up at 25% above

the maker's recommendation for serum electrophoresis. The weighed starch powder was added to half the required total volume of gel buffer (Appendix 6) in a large round bottomed flask and the remaining volume of the buffer used to wash down the starch powder from the side of the flask.

The flask was then heated over a bunsen flame with vigorous swirling, the suspension thickened and when near boiling became less viscous. After degassing, the starch solution was poured to cover a third of the gel forming plate and an even layer made by pushing the spreader (Fig.4b) through the liquid gel to the opposite end, taking with it excess gel. The plate was moved away to prevent flow back of the liquid gel and left undisturbed but covered while the gel set.

3.9.6
Electrophoresis

3.9.6.1
Loading the plates

Lysate beads were thawed in small sample plastic tubes and stood on ice. A line-guide appropriately placed under the gel plate marked the origin for an 8- or 10-slot template to be pressed into the gel. The anodic end of the plate was marked.

Cotton threads (Anchor 6-stranded embroidery threads) slightly shorter than the slots were separated with a fine forceps and absorbed the lysate by capillarity. They were tucked into the gel slots in a known order.

3.9.6.2

Preparation of materials in the electrophoresis tank and electrophoresis

Immediately after loading the gel, the plate was transferred to the electrophoretic tank (Fig. 3) on to a cooling plate at 8° C covered with insulating sheet of polyethylene terephthalate ("Menilex" ICI type gauge 23 mm) and a thin glass plate 15 cm wide covered the central portion of the gel with its edge parallel to the line of the plots.

Wicks ("Spontex" - boiled in Haemosol and rinsed in distilled water) were placed parallel against the thin glass plate while the other end dipped into tank buffer. The wicks conducted the current via the tank buffer through the gel. A thick glass plate was placed over the wicks to hold them in place and prevent evaporation from the gel. The appropriate voltage reading (Appendix 6) was checked across the gel with a voltmeter, any necessary adjustments were made either to the volume of the tank buffer or the power supply setting.

Two plates, one for the specific enzyme and one as a control in case of interfering enzymes, were loaded with identical samples and were run under identical conditions.

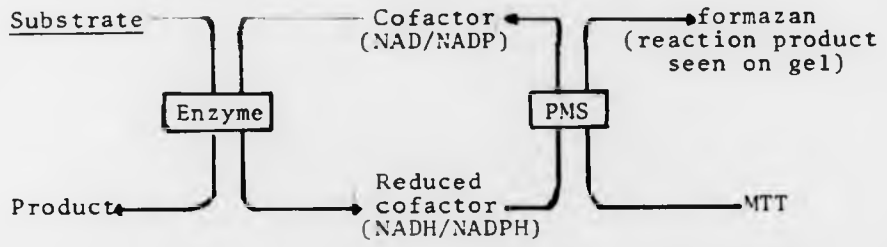
3.9.7

Location and recording of enzyme activity on the gel

After electrophoresis specific enzyme activity was located either by a reaction linked to an insoluble formazan dye or by the oxidation of NADH when bands of non-fluorescing

NAD could be seen against a background of fluorescing NADH under ultra-violet light.

The reaction linked to an insoluble formazan dye can be basically represented as follows:



However, if necessary, other linking enzymes may be used in a chain reaction before linking with the reaction involving reduction of MTT. Areas of enzyme activity on the gel are identified by the presence of formazan a purple staining product of MTT reduction in the presence of catalytic amounts of an electron carrier, phenomethazine sulphate (PMS). The composition of the appropriate reaction mixtures for the different enzymes are shown in appendix table 7.

The fluorescent method; Whatman No. 1 filter papers cut to fit the gel plate were soaked in appropriate reaction mixture (appendix table 7, ASAT and ALAT) without substrate and overlay the gel. The protected gel was then incubated at 37° C for 15 minutes in the dark. The paper was then replaced with another paper soaked in the complete reaction mixture and reincubated. Development was followed every 5 minutes until maximum resolution.

Similar procedures, for both the formazan and

fluorescent methods, for the development of enzymes were carried out, using reaction mixtures without substrate on duplicate plates in order to check for presence of interfering enzymes. Bands detected by both methods were either recorded by photography or by drawing.

CHAPTER 4

ISOLATION, CULTIVATION AND CRYOPRESERVATION OF THEILERIA-INFECTED CELL LINES

4.1 Aims

The aims of experiments carried out in this part of the thesis were as follows:

(i) to establish Theileria-infected cells in culture

(ii) to prepare stabilates from the established cell lines, and

(iii) to define the cooling curve for the cooling procedure used successfully at the EAVRO, Muguga for Theileria-infected cells.

For clarity, this chapter is divided into two sections. Section A deals with isolation and cultivation of parasitized cells while Section B deals with cryopreservation.

4.2 SECTION A - ATTEMPTS TO ESTABLISH THEILERIA-INFECTED CELLS IN CULTURE

4.2.1 Experimental Procedure

4.2.1.1 T. parva (Muguga) in cattle

Steer number L 493 was inoculated with 1.5 ml of a stabilate of infective particles of T. parva (Muguga) and

the animal underwent a lethal reaction. At autopsy, spleen and lymph node specimens were collected within 2 hours of death of the animal and processed for setting up cell cultures within an hour of collection as described in Chapter 3. Two cultures were set up; one from lymph node and the other from spleen cells.

4.2.1.2

T. lawrencei (buffalo paddock) in immunized cattle

Steer numbers L 62 and L 89, and heifer number L 81 were immunized by the infection and treatment method against T. lawrencei. They recovered and were then exposed in the buffalo paddock as described in Chapter 3. Theileria schizonts were detected in all the three animals following exposure in the paddock. Blood and left parotid lymph node specimens from L 81 and L 89 were then collected, processed and used to set up cell cultures. Autopsy spleen specimens were collected from L 62 and used to set up cell cultures. The number of cultures set up from these three animals is indicated in Table XI.

4.2.1.3

T. lawrencei in naturally infected buffaloes

The history of the buffaloes used was given in Chapter 3. Blood was collected from male and female buffaloes in captivity at the Veterinary Department Research Laboratories, Kabete, Kenya. Smears were made from the blood and stined with

Giemsa while serum from each buffalo was tested for immunofluorescent antibodies against T. lawrencei schizont antigen. Mononuclear cells were harvested from the remaining volume of blood as previously described in Chapter 3 and then used to set up cell cultures. The number of cultures set up from each of the buffaloes is indicated in Table XII.

4.2.2

Results

4.2.2.1

T. parva (Muguga) in cattle cells

Spleen impression smears and lymph node cell suspension smears at autopsy had numerous parasites. Only the spleen became established and transformation occurred within 32 days. The lymph node culture was discarded due to contamination with fungi in the first week of cultivation.

4.2.2.2

T. lawrencei in cattle cells

Smears of specimens, used to set up cell cultures, indicated a varying degree of parasitosis as can be seen in Table XI. The information in the table refers to the schizont stage only, regardless of tissue used. There were comparatively fewer parasites in blood specimens than in spleen and lymph node smears. Of the spleen and lymph node smears, the spleen appeared to have more parasites per microscope field. However no attempts were made to count numbers of cells with and without parasites.

In this experiment, cultures number 1, 2 and 3 (Table XI) of L 89 were considered as one culture since they were pooled before transformation took place. Therefore, there were 10 successful isolations out of 13 attempts. The period to transformation in those cultures where isolations were successful varied between 18 days and 33 days. Only one culture of those transforming was used for building up cell stock and cryopreservation from each animal. Culture numbers 2, 3 and 4 of L 81 became contaminated with fungi and were discarded in the first week of the experiment.

4.2.2.3

T. lawrencei in buffalo cells

Table XII summarizes information on the parasitaemia and serology of buffaloes from which blood for cell cultures was collected. No schizont stage of the parasite was detected in blood smears or smears of mononuclear cells. The Table also shows the total number of mononuclear cells harvested from the blood specimens and their distribution to the two types of cultures. Less blood for processing was available from buffalo W 120-76 due to loss of some of the blood during transportation from site of bleeding to the laboratory and as a result only one type of cell culture was set up. Only one out of 13 cultures set up became established and the period to transformation was 27 days. The positive culture was of buffalo number W 325-76 (subsequently referred to as W 325) and was from cells set up in flasks with preformed monolayers.

4.2.3

Discussion

4.2.3.1

T. lawrencei in cattle cells

10 isolations were made out of 13 attempts, using the methods employed by Malmquist et al. (1970), and Malmquist and Brown (1974) for T. parva. Hulliger (1965), the first worker to attempt cultivation of T. lawrencei, cocultivated infected bovine lymphoid cells with a BHK cell feeder layer and modified MEM (Macpherson and Stoker, 1962); she had 5 successful isolations out of 12 attempts. There has been no detailed discussion on the suitability of these different methods for the growth of this parasite. Therefore results in Table XI provided an opportunity of examining success rates achieved here in comparison to those obtained by Hulliger (1965).

Although 10 successful isolations out of 13 attempts seems better than 5 out of 12, there is no strong evidence that these differences are really significant ($P > 0.05$). This suggests that Hulliger's method might be wasteful with medium. Hulliger (1965) used modified MEM containing twice the usual concentration of amino acids and vitamins. The medium was further supplemented with 10% tryptose phosphate broth (2% stock solution) and 10% calf serum. On the other hand, Malmquist et al. (1970) used MEM with normal concentrations of amino acids and vitamins but supplemented their medium with 0.100 gm l^{-1} of L-β-asparagine and 20% FCS. Although Malmquist et al. (1970) did not comment on inclusion

of asparagine (which is not one of the ingredients of Eagle's medium) Brown (personal communication) intimated that medium with asparagine gave better growth than the one without.

Further analysis of the results in Table XI did not provide evidence to show that cultures set up without feeder layers were less likely to establish *in vitro* than those set up with feeder layers. The differences between the proportions of successful isolations for the cultures without feeder layers (4/6) and those with feeder layers (5/7) were not significant ($P > 0.1$). This is not in agreement with observations by Hulliger (1965) who suggested that omission of feeder layers failed to give rise to prolonged multiplication of the infected cells. Malmquist and Brown (1974) found feeder layers desirable in establishment of infected cells but not absolutely essential for maintenance of these cells.

4.2.3.2

T. lawrencei in buffalo cells

Successful isolation of *T. lawrencei* infected buffalo cells was made from only one animal out of the seven captive buffaloes (Table V). Young *et al.* (1978) reported 6 successful isolations out of 19 captive buffaloes and 1 successful isolation from 57 wild buffaloes. The reasons for the low success rates are not known but scarcity of macroschizonts in peripheral blood leucocytes could be partly responsible. The small number of parasitized cells in the initial inoculum could be completely removed from the culture during repeated renewal of growth medium at 3 to 4 day intervals; this inevitably

results in failure to establish infected cell lines. The failure to establish Theileria-infected cell lines from the other six buffaloes (Table XII) did not necessarily mean that those animals were free from harbouring Theileria macroschizonts; indeed the table shows that these buffaloes had piroplasms and in some serological evidence of exposure to Theileria infection at some time in their life.

4.2.3.3

Sera cytoxicity and period of maintenance of cultures

Apart from the various factors discussed above, the type of serum and the length of time cell cultures are maintained might influence the rate of establishing infected cells from the infected tissues.

Cytotoxicity could be an important factor determining whether cells became established or not. Moulton et al. (1971) reported that considerable difficulty was encountered with some sera which were found to be cytotoxic. They therefore tested each batch of serum for growth of standard cell lines in two or three serial passages. All sera used for experiments described in this thesis were tested in this manner. In the report on cultivation of Theileria species Hulliger (1965) did not state whether sera were tested for cytotoxicity, this could have accounted for some of her failures to establish cell lines.

The type and quantity of serum were also reported to be important by Moulton et al. (1971). This however is not easy to assess since no specific experiments comparing media

with FCS, calf serum and adult cattle serum, have been reported. In general calf serum is mainly used in cultivation of T. annulata while FCS is the only type of serum giving consistently good growth with T. parva and T. lawrencei cultures.

The period to transformation for the 10 cultures that became established varied between 18 and 33 days but for each culture there was invariably a period during which parasites could not be detected in Giemsa-stained smears. The apparent disappearance and subsequent resurgence of infected cells were first described by Malmquist et al. (1970). During isolation attempts, therefore, cultures were maintained regardless of apparent absence of Theileria schizonts. It is very tempting to discard cultures once parasites disappear in smears. Hulliger (1965) reported disappearance of the parasite in three cultures on days 30, 43 and 53 of the experiment. She presumably discarded the cultures thereafter. This could have accounted for some of her apparent failures to establish T. lawrencei in culture. Theilen et al. (1968) commenting on isolation of bovine lymphosarcoma cell lines (which are very similar in origin and behaviour in culture to Theileria-infected cells) recommended that cultures should be kept for at least 8 weeks before being regarded as negative. This same reason, probably in part, explains why Tsur et al. (1957) and Brocklesby and Hawking (1958) experienced difficulties in establishing T. parva in culture.

with FCS, calf serum and adult cattle serum, have been reported. In general calf serum is mainly used in cultivation of T. annulata while FCS is the only type of serum giving consistently good growth with T. parva and T. lawrencei cultures.

The period to transformation for the 10 cultures that became established varied between 18 and 33 days but for each culture there was invariably a period during which parasites could not be detected in Giemsa-stained smears. The apparent disappearance and subsequent resurgence of infected cells were first described by Malmquist et al. (1970). During isolation attempts, therefore, cultures were maintained regardless of apparent absence of Theileria schizonts. It is very tempting to discard cultures once parasites disappear in smears. Hulliger (1965) reported disappearance of the parasite in three cultures on days 30, 43 and 53 of the experiment. She presumably discarded the cultures thereafter. This could have accounted for some of her apparent failures to establish T. lawrencei in culture. Theilen et al. (1968) commenting on isolation of bovine lymphosarcoma cell lines (which are very similar in origin and behaviour in culture to Theileria-infected cells) recommended that cultures should be kept for at least 8 weeks before being regarded as negative. This same reason, probably in part, explains why Tsur et al. (1957) and Brocklesby and Hawking (1958) experienced difficulties in establishing T. parva in culture.

4.3

SECTION B Cryopreservation and Resuscitation

4.3.1

Experimental procedure

All suspensions of cell lines L 493, L 62, L 81, L 89 and W 325 isolated in Section A of this Chapter were allowed to grow to high cell concentrations and cryopreserved as described in Chapter 3. Prior to cryopreservation, samples of cell suspensions were withdrawn and used to estimate the viability of cells by the exclusion dye test as described previously in Chapter 3. On a separate occasion, the cooling curves of ampoules containing 1 ml volumes of cell suspensions, fitted with thermocouple probles and sealed with plasticine, were determined by plotting temperature readings against time. To test the viability of cryopreserved cells, resuscitation was carried out twice; first, 24 hours after transfer of ampoules to the liquid nitrogen refrigerator, and again, 7 months after transportation of cryopreserved specimens to the U.K.

4.3.2

Results

4.3.2.1

Cooling curve

Fig. 5 depicts a typical cooling curve for the ampoules at the bottom, in the middle and at the top of a

27 cm cane in the cardboard cylinder in a REVCO at -79° C. The contents of all ampoules supercooled within the first 8 minutes of freezing but the degree of supercooling was greatest in the bottom ampoule and least in the topmost ampoule. The lowest temperatures reached as a result of this supercooling were -16° C, -15° C and -6.5° C for the bottom, middle and topmost ampoules respectively. Following supercooling the temperatures rose to -6° C, -5°C and -4° C for the bottom, middle and topmost ampoules respectively before actual freezing began. Thereafter rapid cooling occurred at approximately 6° C per minute in all samples until a temperature of -40° C was reached. From this temperature onwards the cooling rate was exponential up to the end of the recording of the fall of temperature was stopped. The bottom ampoule cooled fastest reaching a temperature of -78° C within 60 minutes while the topmost ampoule was the slowest reaching a temperature of -68° C in the same period.

4.3.2.2

Viability of cells before and after cryopreservation

Table XIII summarizes information on passage level of different cell lines, total numbers of cells harvested before cryopreservation, viability of cells by the exclusion dye method before freezing and viability of cells after freezing by resuscitation of cells. All cell lines, except cell line L 81, were cryopreserved at passage 4. The total numbers of cells harvested varied from 1.6×10^8 cells for L 81 (a T. lawrencei-infected cattle cell line) to 2.9×10^8

cells for W 325 (a T. lawrencei-infected buffalo cell line). The viability of cells assessed by the exclusion dye test indicated that again cell line L 81 had the lowest number of viable cells (73%) while cell line L 493 (a T. parva-infected cattle cell line) had the highest percentage of viable cells (90%).

24 hours after storage in liquid nitrogen, only cell lines L 62, L 89 and L 493 could be resuscitated without bovine embryonic spleen monolayer cells. Cell lines L 81 and W 325 could not be resuscitated without the feeder layers. moreover, even with feeder layers, L 81 was very slow in re-establishing itself in culture taking approximately 3 weeks while W 325 became fully re-established in culture within one and a half weeks. However, following storage for more than seven months and transportation to the U.K. none of the cell lines could be resuscitated without feeder layers including those cell lines which did not require feeder layers initially (Table XIII).

4.3.3

Discussion

4.3.3.1

Cooling curves

Fig. 5 indicates that there was some degree of supercooling on all the curves. Supercooling is injurious to cells and the lower the temperature falls before actual freezing occurs the greater the injury. This is because the

sudden release of a large amount of latent heat of fusion as nucleation temperature (T) is reached can damage cells in the process. Supercooling can be avoided either by tapping the ampoules with a forceps inside the freezing chamber which enhances the process of enucleation in the sample or by actually seeding samples with frozen medium crystals. The later proposition is impracticable in this case as the ampoules are sealed before cooling and moreover this procedure would interfere with sterility in the sample.

It is not clear why there were differences in extent of supercooling for ampoules at different levels of the REVCO. However, it is probable that opening the REVCO introduced a layer of warm air at the top which raised the temperature at this level of the REVCO close to room temperature. This could have then led to poor conduction of heat away from the ampoule(s) resulting in a slow rate of cooling in this region of the REVCO.

4.3.3.2

Ability of cells to re-establish in culture after cryopreservation

Viability of cells was initially tested by determining the percentage of cells excluding the dye Nigrosin and this appeared to be high (Table XIII, Column 5) but there was no evidence to suggest that variations in the percentages of cells excluding the dye were significantly different ($P > 0.1$). The relationship between viability of cells and ability to exclude nigrosin was first reported by Kaltenbach *et al.* (1958). The high percentages shown in Table XIII can

therefore be regarded as indicative of high viability of the cells used for cryopreservation. However, despite these high percentages, failures in resuscitating cell lines L 81 and W 325 were noted. These failures were however overcome when further attempts to resuscitate cells from these two cell lines were made with feeder layers. In the U.K., at least seven months after all cell lines had been cryopreserved, further failures in resuscitating even those cell lines which did not require feeder layers in E. Africa were observed.

These observations suggest that either the exclusion dye test was not giving accurate assessment of the viability of cells or that the cooling procedure and storage were inadequate in preserving the cells. However, since the thawed cell samples were all available for introduction into culture flasks and therefore no determination of percentage of cells excluding the dye was carried out, it is difficult to assess the extent to which any one of the three factors affected re-establishment of the cells in culture. It is almost certain that improper cooling procedure discussed in the section on cooling curve partially contributed to difficulties in resuscitating the cryopreserved cells.

It is probable that the apparent difficulty experienced in resuscitating cell line L 81 was purely due to incorrect procedures in cooling, thawing and subsequent maintenance in culture - rather than being attributable to very basic differences (e.g. genetic) between this cell line and the other cell lines. There is no strong evidence to suggest that 2 out of 3 and 2 out of 5 (Table XII, last

column) are significantly different ($P > 0.01$).

Although the results indicated some loss in viability of cells, neither evaluation of the cryopreservation procedure nor evaluation of suitability of the exclusion dye test as a measure of viability were actually carried out. For future investigations it is suggested that the method of Lumsden et al. (1966) used to evaluate cryopreservation of Trichomonas vaginalis would be suitable for evaluating these two factors.

CHAPTER 5

OBSERVATIONS ON *THEILERIA*-INFECTED CELL LINES BY LIGHT MICROSCOPY

5.1 Introduction

Hulliger (1965) commenting on *in vitro* cultivation of three species of *Theileria* noted that *Theileria* nuclei in cells multiplied at the same rate as the infected cell and therefore the mean schizont nuclear number (MSN) remained nearly constant. She found no differences between MSNs of *T. parva*, *T. lawrencei* and *T. annulata* schizonts. Irvin *et al.* (1975) used MSN together with percentages of cells in mitosis, percentage of multinucleate cells and percentage of parasitized cells to evaluate short term effects of gamma irradiation on *T. parva*-infected cells. These characteristics are routinely used by the EAVRO, Muguga to evaluate cytotoxicity of batches of sera. Any batch that leads to a marked departure from the known values of the above characteristics for a given standard *Theileria* cell line is regarded as cytotoxic. This, therefore, implies that these criteria are constant for a given cell line.

In this chapter experiments designed to examine the effect of prolonged *in vitro* passage on the cell lines on the above-mentioned characteristics, cultural features and growth patterns were carried out. In addition, chromosome studies of these cell lines were carried out. It was hoped

that any one or all of these factors might provide useful information for characterization of cell lines.

5.2

Experimental procedure

Suspensions of T. lawrencei-infected cells from cell lines L 62, L 81, L 89 and W 325 and those of T. parva-infected cells from cell line L 493 whenever subcultured were set up in 25 ml falcon plastic flasks at 10^5 cells per millilitre in growth medium RPMI 1640. The cultures were passaged at 3 to 4 day intervals, as previously described in Chapter 3, over a period of eleven months.

At approximately 10 subculture intervals, 48-hour old cell suspensions from cultures were centrifuged, then smears were made and stained with Giemsa stain. Smears were then examined for percentage of cells parasitized, percentage of cells in mitosis, percentage of multinucleate cells and the mean schizont number of intracellular Theileria.

On a separate occasion, in between the 10 subculture intervals, daily cell counts were recorded from day 0 to day 4, for Theileria-infected cattle cells, or up to day 5 for Theileria-infected buffalo cells. Results of these counts were used to plot growth curves of various cell lines.

Cultural features of cells in the flasks were examined with an inverted light microscope. Karyotypes were prepared at passage level 4 of the different cell lines and stained with Giemsa stain for examination by light microscopy.

All observations on cell line L 81 stopped at

passage level 60 because this line was lost shortly after passage 63.

5.3

Results

Characteristics of the different cell lines were in many instances similar. The few differences observed in some characteristics appeared not to be related to the *Theileria* species present in the cells. As a result, a general description of the characteristics is given and the minor differences observed are briefly mentioned only when they were noticed.

5.3.1

Cultural features

The five cell lines could be subdivided into three categories on the basis of cell cultural appearance, namely, those that grew as a monolayer, those that grew singly in suspension and those that grew in clumps in suspension. Cells belonging to cell line W 325 grew as monolayer whenever subcultured. This monolayer was firmly attached to culture flasks so that shaking or pipetting usually failed to dislodge the cells from the flask. When the monolayer was completely formed the cells could then appear in the suspension either singly or in clumps.

Cell lines L 89 and L 493 had cells which grew singly but occasionally appeared in clumps of 3 to 4 cells. The majority of cells belonging to cell lines L 62 and L 81

grew in big clumps and appeared like bunches of grapes in suspension, while a small proportion of cells occurred singly in these cultures.

All cells from the different cell lines were refractile but the majority of cells became dull and granular on the third or fourth day of culture. At this time the old medium was replaced by fresh growth medium.

5.3.2

Growth patterns

Cell cultures from different cell lines had sigmoid growth curves (Fig. 6). For individual cell lines, no differences could be detected between the daily cell counts on the 6 separate occasions the cell counting exercise was repeated so the values were combined and the means obtained were used to plot growth curves.

Growth curves for the different cattle cell lines appeared so alike that in this thesis only one curve 'X' (Fig. 6) for cell line L 62 is given to represent the typical growth curves for the other cattle cell lines (L 81, L 89 and L 493). This curve shows that for the cattle cell lines the lag phase was hardly noticeable and was only indicated by the slightly slower growth rate during the first 24 hours. The logarithmic phase was observed between 24 and 72 hours of culture but by 96 hours the percentage of cells excluding nigrosin dye was beginning to decline.

Buffalo cell line W 325 (curve 'Y') followed a similar pattern of growth to that of cattle cell lines except that the lag phase was marked by a definite fall in the number

of cells counted within the first 24 hours. After the fall, the subsequent logarithmic phase and phase of decline were similar to those observed for the cattle cells.

With an initial seeding rate of 10^5 cells per ml, an approximate 10-fold increase in the number of both cattle and buffalo cells was observed within three days. This increase occurred between day 0 and day 3 for the cattle cell lines while it took place between day 1 and day 4 for the buffalo cells (Fig. 6).

5.3.3

Morphology of infected lymphoblasts and Theileria

5.3.3.1

Cattle lymphoblasts

The infected cells were lymphoblasts. Fig. 7 shows that they varied in shape and also varied in size from 10 µm to 14 µm in diameter. There was no evidence of presence of cytoplasmic processes at these magnifications. The cytoplasm was basophilic.

In the majority of cases the lymphoblasts had one nucleus but a small proportion of cells were binucleated. No cells having more than two nuclei were observed. Nuclei were pleomorphic but the majority of them appeared oval or kidney-shaped. They stained intensely and many of them had clumps of chromatin dispersed throughout the nucleus. Figure 8 demonstrates the appearance of nuclear material during metaphase stage of the cell's cycle.

5.3.3.2

Buffalo lymphoblasts

The infected buffalo cells were very similar in appearance to the cattle infected cells except that buffalo cells were smaller in size ranging from 5 µm to 12 µm. The other outstanding feature with buffalo cells was the presence of 'cytoplasmic buds' which stained darker than the rest of the cytoplasm. Fig. 9 shows at least two such 'buds' or herniations on each of the five cells on the left side of the figure.

5.3.3.3

Theileria in cattle lymphoblasts

Macroschizonts were present in the cytoplasm of lymphoblasts but during mitosis they invariably appeared together with the lymphoblast nuclear material as is shown in Fig. 8. They varied in shape and in size, from 4 µm to 10 µm in diameter. In the majority of cells, not in mitosis, the larger the cell the larger the macroschizont appeared to be. Their cytoplasm stained pale blue which was lighter than that of the lymphoblast cytoplasm. Within the schizont cytoplasm were varying numbers of purple staining nuclei which varied in shape and size. Extracellular macroschizonts were also observed (Fig. 10 (arrows)) and their morphology seemed to be well preserved.

Microschizonts were not common in Giemsa stained smears from *in vitro* cultured cells. They were however seen on a few occasions and Fig. 10 shows one of the microschizonts

observed. The figure shows a ruptured microschizont with free merozoites scattered throughout the preparation and probably appearing together with some host cell nuclear material. In the microschizont, the prominent part of individual particles (merozoites) appears to be the intensely staining nuclear material. However, when extracellular, merozoites assumed various shapes atypical of the usual compact spherical shape seen before the rupture. It is not clear whether this variation in shapes also occurs when merozoites are released in vivo or if it is an artifact caused by the strange culture medium environment.

5.3.3.4

Theileria in buffalo lymphoblasts

All features described for the macroschizonts in cattle cells were observed in schizonts in the buffalo cells. However, schizonts in buffalo cells appeared in general to be smaller than those in cattle, similarly their nuclear granules appeared to be much smaller than those in schizonts in cattle cells. No microschizonts were seen in buffalo cells.

5.3.4

Cell line characteristics

Tables XV to XVII summarize data on extent of parasitosis, presence of mitotic and multinucleated cells as well as the average Theileria-nuclei number in the smears of the different cell lines at different passage levels.

The lowest percentage of parasitized cells was 89.2% and occurred with the buffalo cell line while the highest was 99.2% and this was observed in cell line L 493. The

majority of cells not containing parasites were usually smaller than the parasitized cells.

Table IV shows that 1.6% was the lowest mitotic index at any one time during various subcultures and this was observed in smears of cells from cell line L 493, while cells from cell line L 89 had the highest mitotic index of 6.2% recorded during the experiment. The Table also shows that there is no general trend of either an increase or a decrease in the mitotic indices following increasing number of subcultures.

Table XVI illustrates the extent to which multinucleated host cells appeared in smears. The minimum percentage of multinucleated cells was 2.4% observed in smears from cell line L 62 while the highest percentage (11.0%) of multinucleates was seen in smears of cell line L 493. No general trend of either an increase or a decrease in the percentages of multinucleated cells attributable to increasing number of subcultures was seen.

Table XVII shows the mean schizont nuclear number (MSN) of various cell lines at different passage levels. The most striking feature is that the MSN of Theileria schizonts in buffalo cells appears comparatively and consistently smaller than that of MSNs in cattle cells. The Table also indicates that these MSNs were spread over a comparatively small range, despite very wide variations in counts of actual number of nuclei in individual macroschizonts.

5.3.5

Karyotypes

The karyotypes of Bos taurus (2n=60) consists of two sex chromosomes, x and y, which are submetacentrics, the former is the largest while the latter is very small. The remain ing chromosomes are acrocentrics and vary in size.

Fig. 11 shows a typical male cattle karyotype from cell line L 493 with 60 chromosomes. In the figure the only x-chromosome can be seen at approximately 12 o'clock while the y-chromosome, the smallest and with least open arms, lies between 2 and 3 o'clock. Dispersed among the chromosomes are four pale staining Theileria nuclei (Fig.11 Arrows). Similar karyotypic pictures were obtained from cell lines L 62 and L 89.

The karyotype of L 81 with 60 chromosomes is shown in Fig.12 in which two x-chromosomes appear on the left side at 9 o'clock and at approximately 11 o'clock respectively. A third metacentric chromosome on the right side of Fig.12 at 3 o'clock can be seen; this is a marker chromosome for this cell line. Dispersed among the chromosomes are pale staining Theileria nuclei.

Karyotypes of the African buffalo (Syncerus caffer) (2n=52) consists of 8 meta- or sub-metacentric chromosomes, the remainder are acrocentrics including the sex chromosomes. Fig. 13 shows a karyotype from cell line W 325 with 52 chromosomes; the four pairs of meta/submetacentrics can be seen clearly. In the same figure one of the sex chromosomes can be seen (arrow) but the other sex chromosome is difficult

to locate due to extreme spreading out of the arms in some of the acrocentrics. Again Theileria nuclei can be seen although in this figure they appear to be more intensely stained than in the cattle karyotypes.

5.4 Discussion

5.4.1 Cultural features

The significance of different cultural features such as growing singly or in clumps or in a monolayer is not fully understood. These phenomena do not appear to depend on the species of Theileria present. Clumping could be a manifestation of a relatively fast growth rate such that the cells commence mitosis before they are completely separated from sister daughter cells. However, there was no evidence to suggest that the growth rate of the cells lines with clumps was faster than that of cell lines without clumps.

5.4.2 Growth patterns

The relatively long and prominent lag phase of Theileria-infected buffalo cells is interpreted as an indication of the relative difficulty in establishment of the buffalo cells in culture. Probably this phase of growth represents a period the buffalo cells take to attach and form a monolayer on the surface of the culture flask.

However, having created appropriate culture conditions in the flask, the buffalo cells seem to grow as fast as cattle cells. This view is supported by observations on the two growth curves in Fig. 6 in which the slopes of the logarithmic sections of the curves are almost parallel. Further evidence that both groups of cells might be growing at the same rate is indicated by the 10-fold increase in cell numbers within 3 days for both cell types.

5.4.3
Percentage of cells parasitized

Observations in Table XIV are in agreement with those made by Hulliger et al. (1964), Malmquist et al. (1970) and Irvin et al. (1975), in which over 90.0% of the cells present were found to be parasitized. The consistently high percentages of parasitized cells observed over the period of 11-months and the fact that uninfected cells were usually small in size and number lends support to a suggestion by Moulton et al. (1971) that it is Theileria which transforms lymphoid cells into the lymphoblasts and causes the cells to undergo an indeterminate number of replicative cycles.

5.4.4
Percentage of cells in mitosis

In Table XV , the majority of results lie between 2% and 4.4%. This is interpreted as being indicative of a constant rate of multiplication irrespective of increasing number of subculture or different cell line under investigation. This again supports observations by Malmquist et al.

However, having created appropriate culture conditions in the flask, the buffalo cells seem to grow as fast as cattle cells. This view is supported by observations on the two growth curves in Fig. 6 in which the slopes of the logarithmic sections of the curves are almost parallel. Further evidence that both groups of cells might be growing at the same rate is indicated by the 10-fold increase in cell numbers within 3 days for both cell types.

5.4.3
Percentage of cells parasitized

Observations in Table XIV are in agreement with those made by Hulliger et al. (1964), Malmquist et al. (1970) and Irvin et al. (1975), in which over 90.0% of the cells present were found to be parasitized. The consistently high percentages of parasitized cells observed over the period of 11-months and the fact that uninfected cells were usually small in size and number lends support to a suggestion by Moulton et al. (1971) that it is Theileria which transforms lymphoid cells into the lymphoblasts and causes the cells to undergo an indeterminate number of replicative cycles.

5.4.4
Percentage of cells in mitosis

In Table XV , the majority of results lie between 2% and 4.4%. This is interpreted as being indicative of a constant rate of multiplication irrespective of increasing number of subculture or different cell line under investigation. This again supports observations by Malmquist et al.

(1970) and Brown et al. (1976) who observed that cultural conditions being constant there is usually a 10-fold increase in cell numbers over a three day period. Lack of a specific trend in increase or decrease in of the percentage of cells in mitosis suggests that the rate of division of the cells is uninfluenced by the number of subcultures the cells might have undergone.

5.4.5
Percentage of multinucleated cells:

Table XVI shows that percentage of multinucleated cells hardly increased beyond 11.0%. Reasons for occurrence of multinucleated cells and the variation in their percentages are not known. Irvin et al. (1975) could neither demonstrate any specific trend in rise or fall of multinucleated cells nor could they detect differences between tritiated thymidine labelled and non-labelled cultures. Similarly, in Table XVI neither is there a specific trend following increased passage nor are there clearly detectable differences between the cell lines.

5.4.6
Mean schizont nuclear number (MSN)

The fact that MSNs do not appreciably change with subculture was first observed by Hulliger et al. (1964). Results in Table XVII supports those observations and probably indicate the interdependence of both the parasite and the lymphoblast. This interdependence can be disrupted by factors that retard cell multiplication such that the MSN

is considerably increased. These factors could be increased temperature (Hulliger et al., 1966), chemicals like colchicine and Actinomycin D (Hulliger et al., 1966; Moulton et al., 1971b respectively) or gamma-irradiation (Irvin et al., 1975).

Despite the MSN being nearly constant, the results in Table XVII show that there was no clear distinction between MSNs of T. parva and T. lawrencei in cattle cells but that MSNs of T. lawrencei in buffalo cells were distinctly lower. The difference between MSNs in cattle and buffalo cells were significant ($P<0.001$). It is possible that the differences observed might be attributable to presence of a different stock of T. lawrencei in cattle cell lines L 62, L 81 and L 89 on the one hand and another stock of T. lawrencei in buffalo cell line W 325. This would imply that the parasite stock in cell line W 325 would still have a lower MSN even if it were transferred to cattle cells. Alternatively, it is possible that the low MSN of buffalo cells is due to physical limitations on the parasite by the host cell's smaller size (see earlier results in this chapter) and a different chemical environment that might exist in the buffalo cells. If the latter alternative is correct then this would place in doubt the validity of the two species, T. parva and T. lawrencei. Further discussions on this point will be made in Chapter 8.

5.4.7

Karyotypes

Conclusions from the present chromosomes studies are limited since the studies did not include (a) cells from a live uninfected animal, and (b) infected cells at various

passage levels. Cells from live animal would have indicated whether karyotypes had been affected by the presence of Theileria. Karyotyping at various passage levels would have shown if the chromosome number in the different karyotypes would be reduced by increasing passage since Theilen et al. (1968) suggested that there is a tendency of diminution in the number of acrocentric chromosomes with a concomitant formation of meta- and sub-metacentric chromosomes.

Nevertheless, results presented here indicate that additional characteristics for distinguishing Theileria-infected cells can be obtained from karyotypes. On the basis of sex and marker chromosomes and in some cases the differences in total numbers of meta- or sub-metacentric chromosomes it is possible to distinguish between two or more cell lines which are otherwise morphologically indistinguishable. Chromosome studies can therefore be used as a check on possible contamination between two karyotypically different cell lines.

CHAPTER 6

OBSERVATIONS ON THEILERIA-INFECTED CELLS BY ELECTRON MICROSCOPY

6.1 Introduction

A review of the relevant literature revealed a number of reports on the fine structure of *in vivo* *Theileria*-infected lymphoblasts. Of these the most important were by Jarrett and Brocklesby (1966), Buttner (1967), and Mugera and Munyua (1973). Fine structural studies by Moulton *et al.* (1971) and Smith *et al.* (1976) on infected lymphoblasts maintained *in vitro* neither included details of the relationship between parasite and host cell nor compared the three species of *Theileria*, *T. parva*, *T. annulata* and *T. lawrencei*, with a view to finding fine structural differences between them which might serve as additional criteria for differentiating the parasite species. Investigations described in this thesis were carried out to fill in the gaps observed in the reviewed literature.

6.2 Experimental procedures:

Uninfected lymphoid cells were harvested from blood of cow number 745 by the gradient centrifugation method described in Chapter 3, and then processed for scanning and transmission electron microscopy as also described in Chapter 3. Similarly, *Theileria*-infected cell lines L 62, E 174, L 493,

S 3, S 19224 and W 325 were also processed for electron microscopy but W 325 was not included for scanning microscopy.

6.3
Results

6.3.1
Scanning electron microscopy

6.3.1.1
Uninfected lymphoid cells

All cells examined were measuring between 4 and 8 µm in diameter. They were in general spherical and possessed no prominent cytoplasmic processes (Fig. 14). At a magnification of X8000 (Fig. 20), the cell surface was seen to be sponge-like with a cauliflower texture in which dark concavities surrounded by a mesh of criss-crossing string like structures formed an uninterrupted but uneven granular layer. Such cells could easily be distinguished from those infected with parasites.

6.3.1.2
Theileria-infected cattle lymphoblasts

No valid differences were detected between the lymphoid cells infected with the three spp. T. parva, T. lawrencei and T. annulata (Figs. 15 to 17). The possible minor differences noted, involved shape and size which due to the limited number of cells examined could not be taken as distinguishing features. A general description of the surface morphology of infected cells will therefore be given.

Infected cattle lymphoid cells varied greatly in shape and size, the maximum diameter between extreme points of the cell ranging from 9 µm to 16 µm. Fig. 18 demonstrates the typical variation in size observed between cells. In shape, they varied from small spherical to large ovoid or sometimes pleomorphic cells but T. annulata-infected cells were almost invariably spherical irrespective of size. Many cells had prominent long and twisted cytoplasmic processes projecting from their surfaces. The processes were more pronounced on larger cells than on smaller ones and as noted above they were more numerous and longer than those seen in uninfected cells. Moreover cytoplasmic processes were noted to vary in length randomly over the surface of the cells. Fig. 22 shows the base of a cytoplasmic process, while Figs. 23 and 24 show the typical bulbous termination of the processes. Fig. 6 is stereoscopic pair demonstrating how these processes stand away from the cell surface. In some cells, herniation was observed as shown in Fig. 25.

At a magnification of X8000 the surface of these cells appeared smoother (Fig. 21) than the surface of uninfected cells (Fig. 20). The surface of infected cell including that of cytoplasmic processes were covered with uniform sized dots probably gold dust. In some instances there were also pitted holes (Fig. 21) which in some cells were very large (Fig. 20) giving an impression of a dislodged structure in the area, presumably the base of a cytoplasmic process.

6.3.2

Transmission electron microscopy

6.3.2.1

Lymphoid cells from uninfected cattle(Fig. 26,27)

Uninfected cells were of similar size to the cells used in SEM (p.117). Bound by a single cytoplasmic unit membrane, they were, in general, smooth in outline lacking the prominent cytoplasmic processes seen in Theileria-infected cells. There was a comparatively small volume of cytoplasm which had two to three mitochondria in the majority of sections examined. The mitochondria varied in shape and size; they were cristate and bound by double membranes. The Golgi apparatus, when present, was small. At a magnification of X50,000 (Fig. 27) single and double membraned vacuoles (V) probably some of them part of the Golgi apparatus, and also very fine cytoplasmic microtubules (T) were seen. A rough endoplasmic reticulum with scattered ribosomes could also be seen.

In the majority of sections, nuclei occupied between a third and a half of the total cell volume. They were frequently indented in a number of places (Fig. 26) and were bound by a typical double-membraned nuclear envelope. They all had marginated heterochromatin and in Fig. 26 a nucleolus (Nu) can be seen.

6.3.2.2

Theileria-infected cell complex

As there were no observable fine structural differences between the cells infected with the three species of

Theileria or the Theileria spp. themselves a general description of the infected cell, Theileria, and parasite-host cell relationship will be given.

6.3.2.2
The host cell

The striking features of the infected cell were the prominent cytoplasmic processes (Cp); these were projections of the cell membrane enclosing small areas of cytoplasm (Fig. 28). The processes were, however, more conspicuous in the scanning electron micrographs (Figs. 15-17) than in transmission electron micrographs where they often appear fragmented (Fig. 28). The infected cells were larger than the uninfected ones (cf. Figs. 26 and 28). Their cytoplasm had a rough endoplasmic reticulum and in some areas free ribosomes some of which were arranged in clumps. Unlike the uninfected cell mitochondria were more numerous and larger; they varied in shape and were as usual cristate, and bound by double membranes (Fig. 29). They were often swollen possibly an artifact due to fixation (Fig. 31). In the majority of sections, mitochondria were arranged around the parasite mass and host cell nucleus (Fig. 29) but in some sections mitochondria lay between parasite masses (Fig. 28), or between parasite and host cell nucleus.

Single membrane vacuoles were occasionally observed in the interphase cells but vacuolation was most prominent during mitosis (Fig. 32a). The vacuoles varied in size, shape and internal appearance, the larger ones being virtually

empty while some of the small ones contained ill defined membranous structures.

A very active Golgi apparatus, as judged by size and number of budding vacuoles, was present (Fig. 29,30a); in most cases it lay between the parasite and the indentation of the kidney shaped host cell nucleus. In some sections Golgi apparatus activity was extended all around the parasite and host cell nucleus. In a number of sections in the Golgi region there were present a number of very electron dense structures (LY) of varying shape and size (Fig.29) interpreted as lysosomes. Also in the Golgi region, particularly in association with the parasite single lengths of annulate lamella (Al) (Figs. 29 - 31) as a prominent feature were seen. These appeared as alternating blocks of electron dense and translucent material edged by unit membranes, constricting at regular intervals. In one section (Fig.30a,b) where Al were cut in two planes at right angle to each other the striking resemblance to nucleopore of nuclear envelope can be seen. At magnification of X42,000, the details of Al pores are revealed as a ring with one or two central electron dense structures.

Cytoplasmic microtubules were present in host cell cytoplasm in varying numbers depending on the developmental stage of the cell. During interphase these microtubules were rarely seen and in most cases did not seem to have any definite arrangement. However, during mitosis these tubules were usually associated with the parasite (Fig.32b, 32d) and host cell

chromosomes. Fig.32b shows that all microtubules are orientated towards one of the cell centrioles.

Host cell nuclei were frequently indented, kidney shaped and pushed to one side of the cell by the invading parasite as is shown in Figs. 28-30a. They occupied about a third of the host cell, and were surrounded by a typical double membraned envelope. Marginated chromatin was present in the majority of sections.

6.3.2.2.2
The parasite

Three forms of the parasite were observed; namely intracellular schizonts, extracellular schizonts and merozoites.

Intracellular schizonts

These varied in shape and size, and were bound by a single cytoplasmic unit membrane (Fig.30b) which was absent, in some places giving an impression of continuity between parasite and host cell cytoplasm. The unit membrane was in some sections associated with microtubules which extended into host cell cytoplasm and out of the sections prior to their termination (Fig. 33).

The cytoplasm of the parasite was closely packed with ribosomes arranged on a very fine endoplasmic reticulum or in small clumps (Fig. 34). Parasite ribosomes were of approximately the same size as host cell ribosomes. Within this mass of ribosomes were scattered occasional single

membrane vacuoles (PSV) (Fig. 31) whose contents varied from a few fine filaments to amorphous electron dense masses. There were also double membrane vacuoles (Plv) (Fig.30b, 31 which had fine membranous structures similar to the cristae seen in host cell mitochondria.

Where multinucleate schizonts were present, the nuclei were bound by typical double membrane envelopes. Nuclear material (Pn) was homogenous and there was no equivalent to the marginated heterochromatin of the host cell nuclei. There were, however, electron dense structures closely associated with some of the nuclei (Fig. 33) details of such a structure can be recognised as a spindle (Ms) terminating in centrioles (Pc) (Fig.34).

Extracellular schizonts

The morphology of extracellular schizonts was well preserved (Fig. 35). All structures described for the intracellular schizonts were observed in the free form, however, no microtubules were seen extending from the cytoplasmic unit membrane and there was an apparent loss of some of the contents of the vacuoles (Plv) described earlier on in the intracellular schizont.

Merozoites

Fully developed merozoites were observed in only one preparation that of T. parva (Fig.36a,b). They were intracellular and dispersed in the host cell cytoplasm. Taking

into consideration the angle at which they were sectioned, merozoites appeared uniform in size, oval to pear shaped in longitudinal plane or round where sectioned at 90° to the longitudinal plane.

They resembled the motile stages of coccidia and the related organisms of the apicomplexan group in fine structural characteristics. At a magnification of X80,000 (Fig. 36c) some sections of merozoites were seen to be bound by a pellicle which consisted of an outer and inner membrane. Surrounding the outer membrane was a distinct but only moderately electron dense fuzzy, spike-like layer (Sc) similar to the plasmalemma of free living amoebae described by Pappas (1959).

Fig. 36b shows a cross-section close to the apical end of the merozoite indicating presence of a polar ring (Pr) and short sections of associated subpellicular tubules (St). At a magnification of X80,000, in Fig. 36c , more subpellicular tubules can be seen in sections through the middle portion of the merozoite.

Profiles similar to the rhoptries (R) seen in other apicomplexan parasites were observed in many sections of merozoites (Fig. 36b), they were narrow near the apex of the merozoite and bulbous in the remaining portion of the merozoite. The figure also indicates that these organelles varied in shape, size and electron density. In some sections of merozoites three structures representing these organelles are seen while in others there are only two present. All rhoptry-like organelles were unit membrane bound and their

contents homogeneous with occasional electron dense irregular patches, possibly staining artifacts.

Micronemes (Mn) or microneme-like structures were observed in merozoites as small, polymorphic electron dense bodies usually clustered in a specific area within the cytoplasm. Ribosomes were dispersed throughout merozoite cytoplasm and appeared to be approximately the same size and shape as the host cell ribosomes and moderately electron dense but clearly distinguishable from the micronemes.

The nucleus when present in the section possessed typical double membrane nuclear envelope and a prominent nucleolus.

6.3.2.2.3
Parasite-host cell relationship

The apparent continuity of parasite and host cell cytoplasm, the positions of the single layer annulate lamella, the cytoplasmic microtubules and the Golgi apparatus in relation to the schizont have already been described above but additional information, especially in relation to parasite behaviour during mitosis, was provided by sections through lymphocytes infected with T. annulata. Fig. 32a is a section through the centriole area of a lymphoblast in which there is a marked degree of vacuolation which had not been observed previously in interphase electron micrographs of Theileria-infected lymphoblasts. The section also includes short lengths of up to 6 chromosomes (Ch) arranged around the mitotic spindle clearly visible at a magnification of X12,600 (Fig. 32b). Three, possibly four parasites are seen, one

drawn out and electron dense at the narrow end where it is attached to the spindle simulating a chromosome. In Fig. 32d, the spindle tubules extend between the three parasite masses much in the same manner as the single layer annulate lamella did (Fig. 31). At a magnification of X32,000 (Fig. 32c) the presence of the centriole (C) and the associated satellite bodies (S) as well as the convergence of the spindle tubules into the centriolar region are clearly demonstrated

6.4 Discussion

6.4.1 The surface morphology of uninfected and infected cells

Polliack et al. (1974) demonstrated surface morphological differences between B and T lymphocytes of human and murine origin. Their findings were that T lymphocyte surfaces were smoother than those of B lymphocytes which were characterized by numerous cytoplasmic processes of varying lengths. The smoother cells were reported to be smaller than the villous cells. They also reported that a proportion of cells had an intermediate type of surface architecture that it was not possible to classify this latter group by SEM alone.

In this thesis, during investigations on uninfected bovine peripheral blood lymphocytes, the cells observed corresponded with those described as T lymphocytes by Polliack et al. (1974) because there were very few or no processes present. However because of the limited number of cells observed on the nuclepore (polycarbonate) membrane when scanning, the presence

of B cells could not be excluded unless during fixation and critical point drying many cells were washed off the membrane and that such a loss was selective for B lymphocytes. Such a washing off is an unlikely possibility since it is the smoother cells which stayed on the membranes while the villous ones were lost. Alternatively, it could be that the Ficoll-Hypaque system of preparation of uninfected cells affected cell surface architecture such that even those cells with processes became modified. But this is also unlikely, since Polliack *et al*. (1974) found no surface feature differences between lymphocytes prepared with and without Ficoll-Hypaque.

With the infected cells, regardless of *Theileria* sp. present, there were obviously two different cell populations, one having numerous cytoplasmic processes and another with very few or no cytoplasmic processes. It was not possible on SEM to determine which of these cells were of B or T type. Moreover, the larger the cell the more villous it was and vice versa and it seemed as if the differences in size and surface appearance were an indication of age difference; the smaller cells being younger than the larger mature ones. No firm conclusions can be drawn on the differences between uninfected and the infected cells.

To evaluate the significance of differences observed in the results between infected and the uninfected cells future studies using separate population of B and T lymphocytes for scanning and *in vitro* infection with *Theileria* infective particles may be necessary. To confirm the results

obtained from the suggested investigations it may also be useful to investigate presence or absence of surface immunoglobulins (Rouse and Babuik, 1974) on the infected cell.

6.4.2

Internal fine structure of the parasites and the infected cells

The fine structure of intralymphocytic Theileria stages was first studied by Jarrett and Brocklesby (1966) who suggested that the parasite might be undergoing double schizogony and that merozoites were produced by a process of budding from the main mass of the parasite. Further work by Büttner (1967) confirmed that intralymphocytic parasites multiplied by schizogony but produced only one type of progeny, namely the extra-erythrocytic merozoite.

The present study at electron microscope level confirmed and contributed to the following points:

(a) morphology of Theileria schizonts and extra-erythroctyic merozoites

(b) mode of nuclear division in Theileria schizonts

(c) behaviour of the schizont during host cell division.

6.4.2.1

Morphology of Theileria schizonts and extra-erythrocytic merozoites

No fine structural differences were observed between the three species, consequently a general discussion is given.

The stages considered in this discussion are the schizont and the extra-erythrocytic merozoite.

Results presented in this thesis confirmed presence of the following features observed by Jarrett and Brocklesby (1966) and by Buttner (1967b):

(a) the variable shape and size of schizonts

(b) the unit membrane

(c) the close ribosomal packing and the multinucleate nature of the parasite as well as membranous vacuoles of varying sizes and shapes.

However, the present findings differ from those of Jarrett and Brocklesby (1966) and Buttner (1967b) in that, in some instances, apparent continuity of cytoplasm between the host cell and Theileria (Fig. 30b) and presence of microtubules in association with the schizont cytoplasmic unit membrane (Fig. 33) were observed. The significance of discontinuities in schizont cytoplasmic unit membrane is not fully understood. It could be just an apparent loss of membrane due to plane of sectioning or it strongly suggests a free exchange of materials between host cell and the parasite. This may therefore mean that Theileria at this stage in its life cycle is treated just as a host cell organelle and that both the parasite and host cell benefit from products of each other. The importance of microtubules will be discussed in the section dealing with behaviour of the parasite during host cell division.

The fine structure of extra-erythrocytic merozoites was described by Buttner (1967a) who reported that merozoites were bounded by a pellicle of a single unit membrane. Fig. 36b shows merozoites with single unit membranes but merozoites with two membranes, an outer typical unit membrane and an inner less electron dense membrane were also seen. The pellicle formed by two membranes is common in the motile stages of the apicomplexan group of parasites which includes coccidia and babesia. Figure 36b indicate the presence of a polar ring, subpellicular tubules, profiles resembling rhoptries as well as microneme-like structures, all organelles described by Scholtyseck (1973) for coccidia. Such basic organelles have also been observed in merozoites of babesia. These findings lend support to the proposition by Levine (1971) that Theileria is a member of subphylum Apicomplexa even though some features such as micropore and conoid have not yet been demonstrated. Moreover, Scholtyseck (1973) stated that a conoid does not exist in piroplasms.

The variation in shape, size and degree of electron density of rhoptries noted in Fig. 36b is not uncommon in subphylum Apicomplexa. Scholtyseck (1973) noted that rhoptries can vary considerably in shape and that they could be tortuous and occasionally some portions might appear empty hence the variation in electron density. The variation in the number of rhoptries-like organelles in different sections of merozoites seen in Fig. 36b could be due to two reasons. Firstly there might be only one pair of rhoptries but because they are tortuous some sections appearing as a third rhoptry could be a folded portion of one of these organelles. Alternatively,

there might be more than one pair of rhoptries, Scholtyseck (1973) observed that in sporozoites of Eimeria, Toxoplasma, Besnoitia, Sarcocystis, Frankelia, and in some merozoites of Eimeria and of Babesia ovis from ticks had more than one pair of rhoptries.

6.4.2.2

Mode of nuclear division in Theileria schizonts

The discussion below is only concerned with nuclear division of the intralymphocytic schizont since no extracellular schizonts were seen to be dividing. During in vitro culture two processes of division were observed namely the nuclear division which was independent of the rest of the parasite, and splitting up and distribution of the schizont into daughter host cells. The latter form of division will be discussed in the section on behaviour of Theileria during host cell mitosis.

Büttner (1967) was the first to describe binary fission of Theileria nuclei; he observed that there were two electron dense areas with associated tubules from which nuclear division commenced. Fig. 34 shows and confirms presence of these structures. This type of intra-nuclear division was also described by Hammond (1973) for a coccidia schizont Eimeria ninakohlyakimovae. There is a remarkable resemblance between Fig. 38 of E. ninakohlyakimovae by Hammond (1973) and the dividing nucleus of Theileria in Fig. 34 of this thesis. Despite the marked resemblance no distinct centrioles comparable to those of coccidia have been observed for Theileria. It is, nevertheless, felt that the dark, very

electron dense spots just adjacent to the nucleus in some sections of Theileria schizonts (Fig. 33) could be centrioles indicating impending nuclear division. There are also similarities between dividing nuclei in a Theileria schizont and dividing nuclei in a microgamont of genus Eimeria shown in Fig. 19 by Scholtyseck (1973).

6.4.2.3
Behaviour of the schizont during host cell division

The other type of Theileria division involves splitting the macroschizont body and was described by Hulliger et al. (1964). Their findings were later confirmed by Malmquist et al. (1970) and Moulton et al. (1971). Although these workers noted parasite association with host cell mitotic-spindle by light microscopy, they did not demonstrate the fine structural details shown in Figs. 32a-d. The results presented here are therefore important in two ways. First, they confirm light microscopy observations by Hulliger et al. (1964) and Malmquist et al. (1970). Secondly, for the first time, the fine structural relationship between parasite and host cell during mitosis has been demonstrated. The figures show conclusively that the parasite masses are accepted by the host cells as if these parasites were host cell chromosomes. This is a clear indication of the utilization by the parasite of the host cell's facilities.

Hulliger et al. (1964) had suggested that the increased rate of cell division might be due to an association of the parasite with the host cell Golgi apparatus. At that time, their hypothesis could not be examined because no fine

structural studies had been carried out. However, when Moulton et al. (1971) and De Martini and Moulton (1973) reported that the Golgi apparatus was either rarely seen or small, it seemed as if the hypothesis by Hulliger et al. (1964) might not be true. On the other hand, on the basis of results presented in this thesis there is evidence of increased Golgi activity when compared with that of uninfected cells. Part of this increased activity was the formation of a single layer annulate lamella in close proximity to the schizont cytoplasmic unit membrane.

Maul (1968) presented evidence, from studies on human melanoma cells cultured in vitro, suggesting that annulate lamellae (Al) were transformed fenestrated cisternae of the Golgi apparatus. However, other modes of formation of AL from nuclear envelope were detailed by Wischnitzer (1970) who concluded that modes of formation of AL may not necessarily be the same in different cell and species of organisms.

Although Al are frequently observed in embryonic, germinal and neoplastic cells their function remains a matter of speculation. Merkow et al. (1968) thought that they represented a specialized endoplasmic reticulum with a nuclear-cytoplasmic intermediary function. Wischnitzer (1970) concluded that Al were a transitory organelle involved in protein synthesis in cells having unusually high metabolic needs because they occurred periodically and were either associated with ribosomes or they stained basophilically. However, Chemitz and Salmberg (1978) observed that treatment of tumour cells with colchicine or other anti-tubulin agents

reinforced formation of annulate lamellae. They then concluded that Al and nuclear envelope played a role in the control of cytoplasmic microtubule complex (CMTC). In this connection, it is therefore interesting to note that Al were rarely seen at the time cytoplasmic microtubules were present in the Theileria-infected lymphoblasts.

One of the outstanding findings in this study was the presence of massive numbers of cytoplasmic microtubules in relation to the parasite during mitosis. Whether these tubules were of parasite or host cell origin was not determined. However, it was clear that during cell division there was an obvious association between parasite and host cell mitotic spindle (Fig. 32b), the parasite acting like a chromosome being drawn with the spindle contraction into daughter cells prior to cytoplasmic division.

From the known transitory nature of Al and the part played by Al and the nuclear envelope in control of CMTC a hypothesis can now be put forward that Al aids or directs formation of microtubular elements in relation to the parasite cytoplasmic unit membrane while the host cell nuclear envelope directs formation of the microtubule elements in association with the host cell spindle and chromosomes during mitotis. The association of Theileria with the microtubular complex ensures that the parasite is almost invariably carried over into both daughter cells.

CHAPTER 7

ISOENZYME INVESTIGATIONS ON *THEILERIA*-INFECTED CELL LINES

7.1
Introduction

Enzymes are protein substances which catalyse biochemical reactions. An enzyme may act independently of the living cell by which it is secreted but is an essential constituent of it. In addition to the general properties of catalysts, it is highly specific in action, usually heat labile, sensitive to changes in pH and to substances which may act as coenzymes or toxins. Each enzyme is classified according to the type of reaction it catalyses.

Isoenzyme (Isozyme) is a term applied to one of two or more multiple forms of an enzyme that arise from genetically determined differences in primary structure. They are proteins of the different chemical constitution catalysing the same reaction and may or may not be separable by electrophoresis depending on the electrical charge on their outer surface or the molecular size and the gel matrix.

The technique of zone electrophoresis in starch gels was developed by Smithies (1955) and used in genetic studies of serum proteins of man (Smithes and Walker, 1955) and cattle (Smithies and Hickman, 1958). Allen (1960) then used the electrophoretic and histochemical techniques to analyse esterases of the protozoan *Tetrahymena pyriformis* and found that the eserine class of esterases showed differences in different stocks of this protozoa. Later, Reeves and

Bischoff (1968) made a qualitative study of five amoebal enzymes. They were able to demonstrate variations in izo-enzyme patterns within E. histolytica and between various Entamoeba spp. Further work on parasitic protozoa, Plasmodium spp. was by Carter (1970) whose isoenzyme investigations confirmed the distinction between subspecies Plasmodium berghei berghei and Plasmodium berghei killicki but no differences betweek Plasmodium berghei yoelii and P.b. killicki. Carter (1973) found considerable isoenzyme variation among strains of P.b berghei and Plasmodium vinckei, he could therefore identify groups of parasites within each species. The results generally substantiated classical taxonomy but the identification of groups within a species provided further information on their relationships. Again, on the basis of isoenzymes variation it was possible to distinguish between the classically established taxonomic groups of Leishmania (Kilgour et al., 1974; Gardener et al., 1974). These observations together with the development of thin layer starch-gel for demonstrating isoenzyme variants within and between species of trypanosomes (Kilgour and Godfrey, 1973; Bagster and Parr, 1973; Kilgour et al., 1975; Godfrey and Kilgour, 1976) prompted this preliminary investigation into the possibility of characterizing Theileria-infected lymphoblastoid cell lines by isoenzyme variants

The aims of experiments carried out and described in Section A of this Chapter were therefore:

a) to determine if within Theileria-infected cell lines there were any indications of isoenzyme variants in the range of enzymes routinely used in the Department of Medical Protozoology in this school, and

b) to determine which of those enzymes warranted further investigations to improve interpretation of the results for Theileria-cell lines.

7.2

SECTION A - Preliminary experiments

7.2.1

Experimental procedure

The following Theileria-infected cell lines were used: L 62, L 81, L 89, L 493, E 174, W 325 and S 15. Theileria sp. associated with each cell line as well as the history of these cell lines have been given in Chapter 3. Each cell line was pooled in a 250 ml Falcon flask, then the infected cells harvested, and their numbers and viability estimated before being used to prepare lysates for electrophoresis. Electrophoresis of lysates and location of enzyme activity on the gel were then carried out for the following enzymes:

		E C.No.(1972)
(i)	malate dehydrogenase (MDH)	1.1.1.37
(ii)	malic enzyme (ME)	1.1.1.40
(iii)	glucose 6-phosphate dehydrogenase (G6PD)	1.1.1.49
(iv)	glyceraldehyde phosphate dehydrogenase (GAPDH)	1.2.1.12
(v)	aspartate aminotransferase (ASAT)	2.6.1. 1
(vi)	alanine aminotransferase (ALAT)	2.6.1. 2

(vii)	phosphoglucomutase (PGM)	2.7.5. 1
(viii)	aldolase (ALD)	4.1.2.13
(ix)	glucose phosphate isomerase (GPI)	5.3.1. 9

Procedures for electrophoresis and location of enzyme activities on the gel for these enzymes were described in Chapter 3. These procedures were repeated three times for each enzyme in order to confirm the original results. Only the growth medium was used as a control at this exploratory stage; no uninfected lymphoid cells were available at that time. No units of enzyme activity of the lysates were determined before electrophoresis at this stage. All cell numbers were adjusted to 10^8 total cells before being used for preparation of lysates.

7.2.2

Results

7.2.2.1

Viability of cells used for preparation of lysates

The viability of cells used in this experiment ranged from 79.0% to 92.0% but the majority of cells, from the various cell lines, were close to 90.0% (Table XVIII). This indicates that a big proportion of the cells used were viable and therefore suitable for the preparation of lysates.

7.2.2.2

Electrophoretic patterns of enzymes

The results of electrophoretic patterns for the enzymes examined are summarized in Figures 37 to 44 and

(vii) phosphoglucomutase (PGM) 2.7.5. 1

(viii) aldolase (ALD) 4.1.2.13

(ix) glucose phosphate isomerase (GPI) 5.3.1. 9

Procedures for electrophoresis and location of enzyme activities on the gel for these enzymes were described in Chapter 3. These procedures were repeated three times for each enzyme in order to confirm the original results. Only the growth medium was used as a control at this exploratory stage; no uninfected lymphoid cells were available at that time. No units of enzyme activity of the lysates were determined before electrophoresis at this stage. All cell numbers were adjusted to 10^8 total cells before being used for preparation of lysates.

7.2.2

Results

7.2.2.1

Viability of cells used for preparation of lysates

The viability of cells used in this experiment ranged from 79.0% to 92.0% but the majority of cells, from the various cell lines, were close to 90.0% (Table XVIII). This indicates that a big proportion of the cells used were viable and therefore suitable for the preparation of lysates.

7.2.2.2

Electrophoretic patterns of enzymes

The results of electrophoretic patterns for the enzymes examined are summarized in Figures 37 to 44 and

are subdivided into three categories. The first category includes enzymes with differing electrophoretic patterns between the infected cell lines while the second category included those where enzyme activity was detected but was similar for the different cell lines. The third category had no detectable enzyme activity.

7.2.2.2.1
Enzymes where electrophoretic pattern differed between cell lines

These were GPI, GAPDH and ALD.

Fig.37 summarizes the isoenzyme pattern of GPI and indicates that bands A, B and C were common to all cell lines. Band C was slow developing and very faint except for the buffalo cell line. Band C became difficult to develop with increasing time of storage of the lysate.

Variation in isoenzyme patterns was only observed in the fastest moving band, D. Cell line W 325 did not have band D. The different positions of band D are indicated by the Greek subscripts following this letter. Therefore the two cell lines L 81 and E 174 were in position D -α , cell lines L 62, L 89 and L 493 occupied position D -β while only one cell line S 15 was in position D -γ .

Fig. 38 is a summary of the electrophoretic patterns of GAPDH which had two electrophoretic bands; band A was cathodic while band B moved anodally. Band A was similar in all cell lines including the buffalo cell line. Band B is again qualified by the subscripts α, β and γ to indicate the different positions of this band in the different cell lines.

B - α is for the buffalo cell line W 325, while B - β is for the cattle cell line infected with T. annulata. B -γ indicates the position of this isoenzyme for the various cattle cell lines infected with either T. parva or T. lawrencei.

Fig. 39 shows that ALD varied from three to four electrophoretic bands depending on the cell line used. Band A was common to all cell lines. Bands B and D were also common to all cell lines while band C was only common to the cattle cell line infected with T. annulata and the buffalo cell line with T. lawrencei. However, bands C and D for the buffalo cell line were diffuse and almost continuous unlike those of T. annulata-infected cattle cells.

7.2.2.2.2

Enzymes where patterns were similar for the different cell lines

This group included PGM, MDH, ASAT, ME and G6PD. The three electrophoretic bands A, B and C of PGM were all anodic (Fig. 40). The slow moving band stained intensely while the other two bands were faint and tended to disappear with prolonged storage of lysate beads. As a result, bands B and C could not be detected on subsequent electrophoretic runs.

There were two MDH isoenzymes represented by two bands (Fig. 41), one anodic and the other cathodic, both bands were of the same intensity on staining. These bands developed within 30-45 minutes.

There were two ASAT bands (A and B), one anodic and the other cathodic (Fig. 42). These bands were very

diffuse and usually difficult to localise. On different electrophoretic runs the bands appeared in slightly different positions but four trials convinced the author that there were no differences in electrophoretic mobilities of the bands for the different cell lines and the apparent variations observed were probably due to unevenness of the starch gel.

ME had only one electrophoretic band, A, for all the cell lines examined (Fig. 43). The band was very slow developing during staining and was usually very faint even after 3 hours of incubation at 37° C and subsequent overnight

G6PD had one anodic diffuse band A; the intensity and width of the band from enzyme extracts of buffalo cell line W 325 was greater than that observed for cattle cell lines (Fig. 44).

7.2.2.2.3

Enzyme where no electrophoretic pattern was detected

There was only one enzyme, ALAT in this category. Attempts to locate bands of enzyme activity were tried unsuccessfully on three occasions; the third time a freshly prepared lysate, which was different from the one used during the first two attempts was used. No enzyme was demonstrable on all the three occasions. As no ALAT assays had been attempted to determine units of enzyme activity in the lysates, it was not possible to conclude whether failure to detect ALAT was due to lack of activity or that conditions for the

diffuse and usually difficult to localise. On different electrophoretic runs the bands appeared in slightly different positions but four trials convinced the author that there were no differences in electrophoretic mobilities of the bands for the different cell lines and the apparent variations observed were probably due to unevenness of the starch gel.

ME had only one electrophoretic band, A, for all the cell lines examined (Fig. 43). The band was very slow developing during staining and was usually very faint even after 3 hours of incubation at 37° C and subsequent overnight

G6PD had one anodic diffuse band A; the intensity and width of the band from enzyme extracts of buffalo cell line W 325 was greater than that observed for cattle cell lines (Fig. 44).

7.2.2.2.3

Enzyme where no electrophoretic pattern was detected

There was only one enzyme, ALAT in this category. Attempts to locate bands of enzyme activity were tried unsuccessfully on three occasions; the third time a freshly prepared lysate, which was different from the one used during the first two attempts was used. No enzyme was demonstrable on all the three occasions. As no ALAT assays had been attempted to determine units of enzyme activity in the lysates, it was not possible to conclude whether failure to detect ALAT was due to lack of activity or that conditions for the

development were unsatisfactory or that in Theileria and in the infected cells the enzyme is unusually labile.

Future studies on this enzyme should therefore begin with estimating units of enzyme activity in all lysates and attempting to develop the best conditions for activity before electrophoresis.

7.2.3
Conclusions

The results from enzymes with similar electrophoretic patterns for the different cell lines indicated that these enzymes were unlikely to be profitable in the search for differentiating characteristics between Theileria-infected cell lines. Therefore no further investigations were carried out on these enzymes.

However, the results obtained with GPI, GAPDH and ALD seemed promising but required control cells and standardization of the enzyme activities in order to have meaningful interpretations of variations in the isoenzyme electrophoretic patterns.

No further growth medium controls would be required in the subsequent investigations as there were no detectable patterns specifically relating to the medium.

7.3
SECTION B - Further experiments with three selected enzymes

In Section A, the need to include control cells for meaningful interpretations of the electrophoretic patterns

of GAPDH, ALD and GPI was noted. It was however difficult to obtain satisfactory control cells since fresh uninfected lymphoid cells from cattle or buffalo do not have the same ability to undergo unlimited multiplication *in vitro* like *Theileria*-infected lymphoid cells. To simulate a situation similar to that prevailing in *Theileria*-infected cells, mitogens capable of stimulating both B and T lymphocytes were used on uninfected cells. Later during these investigations, 3 lymphoblastoid cell lines, uninfected with *Theileria* but capable of unlimited multiplication in culture became available and were therefore regarded as the best controls and therefore used as the controls for *Theileria*-infected cell lines.

The aim of experiments carried out in this section was to determine if variations in electrophoretic patterns observed in Section A could have been due to any one or a combination of the following: (a) lack of standardization of activities in lysates before electrophoresis, (b) age, sex or breed of the host cell, (c) overstimulation of host cell biochemical processes by *Theileria*, and/or (d) *Theileria* species differences.

7.3.1
Experimental procedure

Lymphoid cells were harvested from blood and lymph node samples of animals shown in Table VI. The viability of these cells was determined before setting up cell cultures for mitogenic stimulation. Estimations of uptake of tritiated thymidine were made three days after the cultures had been

set up. Stimulated and non-stimulated cells from macrocultures (Chapter 3) which were not pulsed with thymidine were then harvested for preparation of lysates. The procedures used in this section were described in detail in Chapter 3.

Theileria-infected cell lines and cell lines not infected with Theileria (B1-3, 20 and 24) were allowed to grow up to about 2×10^8 cells and were then harvested and the cell number adjusted to 10^8 total cells for the preparation of lysates. Samples for determining the viability of all cells and for making smears to determine cell line characteristics for the infected cells were withdrawn before lysate preparation.

Using methods already described in Chapter 3, the protein content and enzyme activities of various cell lysates were estimated. Electrophoresis was then carried out using lysates adjusted to similar units of enzyme activity with 5% BSA for GPI and ALD enzymes only. No determinations of enzyme units for GAPDH were carried out and therefore there were no adjustments of enzyme activities for this enzyme before electrophoresis.

7.3.2

Results

7.3.2.1

Viability and uptake of tritiated thymidine by uninfected lymphoid cells

The viability of all lymphoid cells from blood and lymph node samples was over 80.0% except for samples from

calf blood, where it was 77.0% (Table XIX). This suggests that the majority of cells were viable and it was therefore assumed that the cells were suitable for mitogenic studies.

The table also shows that, in general, there was a greater stimulation of the cells by Con A than by LPS; the stimulation indices for Con A stimulated cells are higher than those for LPS. The fact that the various samples could incorporate tritiated thymidine after 3 days of incubation in culture, was a good indication that these cells were still viable. Consequently, it is presumed that these cells were suitable for use in preparation of lysates for control purposes.

7.3.2.2

Viability and characteristics of various lymphoblastoid cell lines

A big proportion of cells from the different cell lines were viable according to the exclusion dye test (Table XX). The characteristics of Theileria-infected cell lines in the last four columns of Table XX are similar to those observed in Chapter 5. It is therefore concluded that the samples used for preparation of lysates were viable and a fair representation of the various Theileria-infected cell lines.

7.3.2.3

Protein content and enzyme activity of lysates

There were no indications that levels of protein content or enzyme activity depended on mitogenic stimulation (Table XXI). Similarly, in Table XXII, there were no indications that levels of protein content or enzyme activity were influenced by the presence or absence of Theileria in the cells. However, there were indications that levels of specific enzyme activities were higher in established cell lines (Table XXII) than in lymphoid cells from primary cultures (Table XXI).

7.3.2.4

Electrophoretic patterns

7.3.2.4.1

GPI (Fig. 45)

Bands A, B and C were common to all cells. Band D-α was found both in T. lawrencei and T. parva-infected cattle cells of cell lines L 81 and E 174 respectively. D-β was present in two T. lawrencei and one T. parva-infected cattle cells of cell lines L 62, L 89 and L 493 respectively while band D-γ was found in T. annulata-infected cells of cell lines S 3, S 15 and S 19/224. No D band was observed in the T. lawrencei buffalo cells from cell line W 325.

On the basis of these results, GPI isoenzyme patterns for the various cells could be put into 4 sets (Fig. 45). Set 1 included not only the uninfected cells of cattle and buffalo but also the only infected cells of buffalo (W 325). Set 2 included cells from cell line L 81 with a parasite designated T. lawrencei and those from cell line E 174 with

a parasite designated T. parva. Set 3. included cells from cell lines L 62 and L 89 with parasite designated T. lawrencei and those cells from cell line L 493 with parasite designated T. parva. Set 4. was exclusively for cells with parasites designated T. annulata.

7.3.2.4.2
GAPDH (Fig. 46)

Band A was common to all cells. Band B was only present in the infected cells but showed three variations. B - α was found in the only buffalo infected cells while B - β was common only to T. annulata-infected cells. Band B - γ was present in both T. lawrencei and T. parva cattle cell lines only.

As a result of these findings electrophoretic patterns of these cells could also be divided into four sets. Set 1. included all uninfected cattle and buffalo cells irrespective of whether they were stimulated or unstimulated; it included also cell lines BL-3, 20 and 24. Set 2. included all those cells infected with either T. lawrencei or T. parva. While Set 4. included all the cells from the three T. annulata cell lines.

7.3.2.4.3
ALD (Fig. 47)

Band A was common to all cells. Band B was found in all infected cells, the mitogen stimulated cells, cells from cell lines BL-3, 20 and 24 which multiply in culture like cells infected with Theileria. It was not found in normal buffalo and cattle lymphoid cells. Band C was present

only in T. annulata-infected cells but a diffuse band in a similar position was found in buffalo cells infected with T. lawrencei. Band D was common to all cells except the control buffalo lymphoid cells. A diffuse band D was found in buffalo cells infected with T. lawrencei.

The results obtained could therefore be grouped into five sets. Set 1. included all uninfected cattle cells while Set 2. included uninfected buffalo cells. Set 3. included all mitogen stimulated uninfected cattle cells, cells from cell lines B1-3, 20, 24 and all cells infected with either T. lawrencei or T. parva. Set 5. included all cells infected with T. annulata.

7.3.3

Discussion

No detailed discussion of the results in Tables XXI and XXII will be given since the aim in determining the enzyme units of each lysate was simply to enable appropriate adjustments to be made such that all samples would have similar units of activity before electrophoresis.

Consequently the only point of interest in relation to the isoenzyme investigations is that lysates from Con A stimulated cells, Theileria-infected cells and cells from cell lines BL-3, 20 and 24 had higher units of enzyme activity per ml than lysates from unstimulated cells. This observation appears to suggest that Theileria stimulates lymphoid cells like Con A does, unless all of this increased activity could be shown to be due to the parasite enzyme alone. Similarly cell lines B1-3, 20 and 24 appear to be

in a similar hyperactive state but the stimulation in this instance is unknown; it could be a virus although no such virus has been identified in these cells as yet.

7.3.3.1

Electrophoretic patterns

From the results of electrophoretic patterns of the three enzymes, all cells infected with T. annulata distinctly and consistently belonged to one group. Similarly, the only buffalo cells infected with a parasite designated T. lawrencei were consistently different from all cattle cells infected with Theileria, including even those cells infected with a parasite designated as T. lawrencei. However, the results indicate that cattle cells infected with either T. lawrencei or T. parva could not be distinguished by isoenzyme electrophoretic patterns of ALD (Fig. 47) and GAPDH (Fig. 46). The variations observed with GPI for cattle cells infected with either T. lawrencei or T. parva did not correspond with the designations of the parasite species supposedly present in the cells. The significance of these variations is, therefore, not clear especially since these variations were not only between cattle and buffalo cells. An attempt is therefore made below to try and explain the possible significance of some of these variations.

7.3.3.1.1

Differences between infected cell lines

Preparation and electrophoresis were carried out

under identical conditions for all lysates and the possible cause of isoenzyme variations in the infected cells could have been due to one or more of the following:

(a) differences between buffalo and cattle cells,

(b) differences in sex and/or breed of the cattle from which infected cells were isolated,

(c) presence or absence of different isoenzymes during different phases of the cell cycle,

(d) differences between species and/or stocks of the parasite within the cells.

The differences between host cells probably account for the variations in electrophoretic patterns between cattle cells (L 62, L 81 and L 89) infected with T. lawrencei and buffalo cells (W 325) infected with T. lawrencei (Figs. 45 to 47). This is however on the assumption that the parasites in both the cattle and buffalo cells were the same species and of the same stock. Although the parasites were designated T. lawrencei in the above-mentioned cell lines it is possible that the buffalo parasite might be different from the cattle parasites. The difference could be explained by an existence of different parasite species or strains in the different areas from which animals harbouring the parasites were captured. The parasite in cell lines L 62, L 81 and L 89 is presumed to have been derived from the buffalo paddock at Muguga. This parasite in the paddock is believed to have originated from Laikipa District, northern Kenya where the two buffaloes in the paddock originated (Young et al., 1977). On the other hand, buffalo W 325 was captured in the Mara region of Kenya which is in the South.

It seems unlikely that the differences observed could have been due to sex since cells of L 81, a Friesian heifer had a similar electrophoretic pattern with those of E 174, an Ayrshire steer. It is also unlikely that differences in electrophoretic patterns were due to breed differences since as indicated above, L 81 and E 174 were of different breeds. Further indications that the differences were probably not due to breed are that L 493, L 89 and L 62 had a different pattern from that of E 174 although all the four animals were of the same breed (Table IX). The differences between electrophoretic patterns of S 3, S 15, S 19/224 on the one hand and that of L 81 on the other furthere suggest that variations were not due to breed differences since again all of these cattle were Friesians.

The differences observed in the electrophoretic patterns of the various cell lines could also have been due to presence or absence of some isoenzymes during certain phases of the cell cylce. To investigate this properly it would require synchronous development of all cells in cultures but this was beyond the experimental capabilities of the author. However, a less satisfactory alternative of harvesting cells on different days of culture was used. No differences were observed between the patterns of cells harvested on days 1, 2, 3 and 4. It therefore seems unlikely that the differences were due to switching on or off of certain isoenzymes at different developmental stages of the cells.

7.3.3.1.2

Similarities in patterns between cattle cells infected with either T. parva or T. lawrencei

Discussions in the previous section appear to suggest that the variations in electrophoretic patterns (at least with cattle cells) were likely to have been due to presence of the different Theileria spp. rather than host cell differences.

The isoenzyme patterns of ALD and GAPDH were similar for cells infected with either T. parva or T. lawrencei. This suggests that either both these species have genetic information in common or it is by coincidence that these parasites have enzymes with similar electrophoretic mobilities; de Lima et al. (1979) have demonstrated such a coincidence with T. cruzi-like flagellates.

However, with GPI the pattern for cells from cell line L 81 (with a parasite designated T. lawrencei) and cell line E. 174 (with a parasite designated T. parva) were similar but different from the pattern for cells from cell lines L 62, L 89 (with parasites designated T. lawrencei) and cell line L 493 (with a parasite designated T. parva). These results could be providing evidence for subspeciation within the T. parva - T. lawrencei complex.

7.3.3.1.3

Similarities in electrophoretic patterns of uninfected and infected buffalo cells

Although the patterns of buffalo cells infected with

T. lawrencei were different from those of uninfected buffalo cells for ALD and GAPDH, there were no detectable differences between the infected and uninfected cells with GPI.

The fact that buffalo uninfected cells and T. lawrencei-infected buffalo cells had exactly the same GPI isoenzyme pattern, while with cattle cells the results were different, is probably an indication of how well T. lawrencei parasites have become adapted to the environment in the buffalo lymphoid cells. T. lawrencei infections in buffalo are generally asymptomatic. This is in contrast to the fatal infection in cattle. It seems therefore that the parasite is well adapted to the host cell environment (at least for this enzyme) so that it either makes use of host cell enzymes or produces exactly similar enzymes which are indistinguishable from the host cell GPI isoenzymes by the present investigational methods. However, it would require use of more control and test samples before it can be firmly concluded that uninfected and Theileria-infected buffalo cells have exactly the same GPI isoenzymes.

CHAPTER 8

GENERAL DISCUSSION

At the beginning of these investigations the objectives were to establish in culture lymphoid cells infected with Theileria and subsequently to study the characteristics of such cells in the hope that the investigations would improve our knowledge of the parasite-host cell relationship and the ability to distinguish between some Theileria species affecting cattle. The extent to which these objectives were achieved and the significance of the present findings will be discussed in this chapter.

In Chapter 4, a description of how five cell lines infected with Theileria species were successfully established in culture was given. These results have already been discussed in the same chapter in relation to the results of other workers.

In addition to the specific discussions in Chapters 5, 6 and 7, the results of these chapters, as a whole, will now be examined with regard to the relationship of Theileria with other protozoa at higher taxa, the parasite species and the nature of the parasite-host cell relationship. The significance of this relationship in chemotherapy and the apparent absence of microschizonts in cells maintained in culture will also be discussed.

8.1

Relationship of Theileria with other organisms at higher taxa

The occurrence of Theileria intracellularly and the remarkable resemblance in morphology (schizont intranuclear division and merozoite structure - Chapter 6) and schizogony (Schein et al., 1978) to coccidia and coccidia-like organisms strongly suggests that these protozoa are more closely related than hitherto realized.

Mehlhorn and Schein (1977) and Mehlhorn et al. (1978) have confirmed observations by Cowdry and Ham (1932) and Sergent et al. (1936) that sexual reproduction takes place in T. annulata. Their findings appear to provide another common feature between Theileria and Coccidia. This is in contrast to the original belief (Levine, 1970) that organisms in class Piroplasmea (Levine, 1961) only had asexual reproduction while those of class Sporozoea (Leuckart, 1879) which includes coccidia had both sexual and asexual reproduction. These observations by Mehlhorn and Schein (1977) and those in the foregoing paragraph lend support to the placement of Theileria into the new subphylum Apicomplexa nov. subphylum (Syn., Polannulifera Levine, 1969).

However, there is a need to confirm the results in Chapter 6 especially with regard to the merozoite which provide the main structural characteristics responsible for including Theileria in subphylum Apicomplexa. This is because observations made in Chapter 6 were based on the only single preparation in which merozoites were found. The observations in Chapter 6 and by Schein et al. (1978)

that Theileria merozoites might have subpellicular tubules and a polar ring are not in agreement with the statement by Barnett (1977) that intraerythrocytic merozoites (piroplasms) do not have subpellicular tubules or a polar ring. It is therefore not clear whether these structures are lost upon entry into the erythrocyte.

8.2
Theileria species

In the results reported in Chapters 5, 6 and 7, only isoenzyme variation seemed to agree with the classical speciation in that cells infected with T. annulata had a different isoenzyme pattern from that of cells infected with either T. parva or T. lawrencei. This is on the assumption that the variations observed were due to the parasite alone. There was evidence to support this assumption by at least two enzymes GPI and GAPDH where the extra bands, not observed in the uninfected cells, were seen.

Isoenzyme investigations, however, did not distinguish between T. parva and T. lawrencei. Therefore, in this respect, the results are similar to those obtained with the IFA test by Burridge and Kimber (1973) who found complete cross identity between T. parva and T. lawrencei. The isoenzyme findings are also in agreement with the observations in Chapter 5 where T. parva and T. lawrencei in cattle cells could not be distinguished on the basis of MSNs or other cell line characteristics such as the percentages of cells parasitized, percentages of cells in mitosis or

percentages of multinucleated cells. This lack of distinguishing features between T. parva and T. lawrencei in cattle cells was further confirmed on fine structural studies.

It is not at all certain if the lack of isoenzyme variation between cattle cells infected with T. parva and T. lawrencei came about as a result of passaging cells in culture. In Chapter 2, it was noted that Brocklesby (1966) found that a Kenyan strain of T. lawrencei and a South African strain of T. lawrencei became indistinguishable on passage when such criteria as macroschizont size, percentage of lymphoid cells with schizonts and extent to which microschizonts and piroplasms appeared in smears were examined. Young et al. (1973) made similar observations on a Theileria species pathogenic to cattle and isolated from a buffalo in Tanzania. However, it seems unlikely that there was a change in the isoenzyme patterns due to in vitro passage of the infected cells since cell lines E 174 (with T. parva) at passage 250 and cell line L 81 (with T. lawrencei) at passage 30 had similar isoenzyme patterns for GPI, GAPDH and ALD. No variations were observed between the three Iranian cell lines infected with T. annulata with different degrees of virulence (Table IX). It is therefore unlikely that the variations in isoenzyme patterns observed between some cell lines infected with either T. parva or T. lawrencei indicate parasite ability to cause or not to cause severe disease. Further work to enable firm conclusions on isoenzyme variation regarding the various Theileria species is necessary. Further investigations should first aim at

conclusively confirming that the variations reported in this thesis are due to Theileria. This might require isoenzyme studies on lymphoid cells from an uninfected animal before and after infection by the in vitro infection method of Brown et al. (1973). Similar studies with the piroplasm stage of the parasite derived from infected blood might be useful; the intraerythrocytic stages of Plasmodium species have been used by Carter (1970, 1973) to demonstrate isoenzyme variations between and within species.

8.3

The nature of the host-parasite relationship

It is strange that this relationship in which the parasite is apparently treated as host cell genetic material during mitosis, no reduction in the number of host cell chromosomes was observed (karyotyping observations carried out randomly in the course of maintenance of the cultures but not presented in the thesis showed no change in the number of chromosomes per karyotype). As a result of this observation, it is not at all clear whether the schizont merely gets entangled in the web of the spindle or it is actively involved with the formation of the spindle. It seems unlikely that this association is purely by accident since Hulliger et al. (1964) observed the association of the parasite with host cell spindle in 146 out of 147 cells. This observation together with the consistently high and

almost constant number of parasitized cells in culture suggest that the parasite is in some way actively involved with the host cell spindle formation probably by means of association with the Golgi apparatus as proposed in Chapter 6.

Apart from the apparent close fine structural relationship, there are indications in Chapter 7 (Figs. 43 and 44; possibly also 37 to 42) that there might also be a biochemical relationship between the parasite and the cell. It is however not known whether the parasite and the cell produce identical enzyme simultaneously or whether in this case the enzymes are produced by the cell and then shared with the parasite. Lack of isoenzyme variations (Figs. 43 and 44) even in presence of different Theileria species in the cells supports the latter view. This close biochemical relationship may, however, not be extended to all enzymes operative in this parasite-cell comp-ex; results of Figs. 45 and 46 support this observation. These observations are further corroborated by the views of Irvin and Stagg (1977) who suggested that thymidine kinase (TK, EC 2.7.1.21) is present in both the cell and the parasite but that hypoxanthine guanine phosphoribosyl transferase (HGPRT, EC 2.4.2.8) might be present, at a reduced activity, in the cell but is completely absent in the schizont.

8.4

The significance of the parasite-host cell relationship

The apparent acceptance of the schizont as a host cell organelle (Chapter 6) and the apparent existence of

enzymes either common to both the host cell and the parasite or produced by the cell or the parasite and then subsequently shared between the two, give some indications as to why it has been difficult to find an effective drug against Theileria. Neitz (1957) and McHardy et al. (1976) gave long lists of drugs which were not effective against these parasites. McHardy et al. suggested that the metabolism of T. parva is closely related to that of the host cell and that the difficulty in finding a compound which will exploit differences between the parasite and its host cell might indicate why no effective therapeutic compound was available for ECF.

However, discussions in this Chapter have suggested that there are some differences between some biochemical processes of the parasite and the host cell despite the apparent close relationship. This was indirectly suggested by the existence of extra GPI and GAPDH (Figs. 45 and 46) isoenzymes in Theileria-infected cells. A detailed study of the specific GPI and GAPDH isoenzyme involved may in the future be helpful in the search for a drug with a selective action against the parasite; observations on folate antagonists (Irvin and Stagg, 1977) support this view.

Also observations on parasite-host cell relationship indicate that although the schizont seems to be accepted as a host cell organelle this self deception may not continue with subsequent stages in the life cycle of the parasite. There is, in fact, evidence to suggest that this is so. Some reaction on the part of the host cell or the

parasite in the merozoite stage can clearly be seen in Fig. 36c where the presence of a glycocalyx-like fuzzy coat may be interpreted as parasite secretion or host cell antibody or again as antigen-antibody complex.

8.5

Scarcity of microschizonts in cultures

It was noted in Chapters 5 and 6 that merozoites were rarely observed in either Giemsa stained smears or in sections of parasitized cells examined by transmission electron microscopy. The above observations are in agreement with those of Brocklesby and Hawking (1958) who could not demonstrate macroschizonts breaking up into merozoites in smears of infected cells from cultures. Hulliger et al. (1966) made similar observations and attempted to induce microschizont formation by raising the temperature of the cultures. They were of the opinion that appearance of parasites with increased nuclear particles was evidence for microschizont formation. It is not clear whether what Hulliger et al. (1966) observed were microschizonts or just an increased schizont nuclei number.

With the fine structure results in Chapter 6, it is not at all surprising that microschizonts are not normally observed in smears of infected cells from cultures. The parasite at this stage appears to be accepted as a host cell organelle and in fact micrographs show that it is accepted as a host cell chromosome during mitosis. This then implies that the parasite can go on living

indefinitely as a host cell organelle as long as the cell lives and continues dividing. This relationship is apparently undisturbed *in vitro* probably because the continued replenishment with fresh growth medium ensures that toxic products do not accumulate in the culture medium and the essential nutrients are present.

This relationship is however disturbed by increased temperatures, chemicals, gamma-irradiation or other agents that may interfere with host cell multiplication. The schizont-host cell relationship is disturbed *in vivo* presumably by the host immune mechanism. In this instance, the host immune mechanism might regard the infected cell as a foreign body probably because such cells might secrete parasitic antigenic substances on their cell surfaces. It might then be possible that the formation of micro-schizonts begin.

8.6
Conclusions

In conclusion, observations in this thesis support earlier observations on the relationship of *Theileria* with the host cell by Hulliger *et al.* (1964) and Hulliger (1965). The observations on fine structure have, in particular, aided in appreciating some of the reasons why merozoite formation *in vitro* and effective chemotherapy are hard to come by. The isoenzyme results have indicated that GPI and GAPDH may be useful as a means of differentiating between some *Theileria* species and possibly subspecies.

Table I : A summary of names of diseases and of geographical distribution of various cattle Theileria parasites

Parasite	Name of disease	Geographical occurrence
T. parva	East Coast Fever (ECF)	Africa South of the Sahara, except West Africa
T. lawrencei	Corridor disease	Africa South of the Sahara, except West Africa
T. annulata	Mediterranean Coast Fever or Tropical theileriosis	Mediterranean region, the Middle East up to India, including Southern USSR
T. mutans	Benign bovine theileriosis	Worldwide occurrence
T. sergenti	Theileriosis	Eastern USSR, Japan and Korea

Table II : Placement of *Theileria* and *Babesia* in the higher taxa between 1961 and 1971

Year	Subphylum	Class	Order	Family
1961		Piroplasmasida	Piroplasmorida	Babesiidae Theileriidae
1964	Sarcomastigophora	Piroplasmea	Piroplasmida	
1968	Sporozoa	Telesporea	Eticoccida	Babesiidae Theileriidae
1968		Piroplasmea	Piroplasmida	Babesiidae Theileriidae
1969	Polannolifera	Piroplasmea		
1970	Apicomplexa	Piroplasmea		
1971	Apicomplexa	Piroplasmasida	Piroplasmorida	Babesiidae Theileriidae Dactylosomatidae

Table III : The various genera to which Theileria species have been assigned since 1904

Parasite as known before	New recommended designation	Reason(s)	Author(s)
P. annulatum	Theileria annulata	Presence of lymphocytic Stage	Bettencourt et al. 1907
P. parvum	T. parva		
P. mutans	T. mutans	Resemblance of erythrocytic Stages to T. parva and although no lymphocytic Stage had been observed	France (1909)
P. mutans	Gonderia mutans	No lymphocytic Stage	Du Toit (1918)
G. mutans	Babesia mutans	?	Wenyon (1926)
B. mutans	T. mutans	Lymphocytic Stage demonstrated	Theiler and Graf (1928)
T. mutans	G. mutans	Both multiplied by binary fission in erythrocytes and by schizogony in lymphocytes	Neitz and Jansen (1956)
T. annulata	G. annulata		
G. mutans	T. mutans	Family Gonderidae and genus Gonderia invalidated	Neitz (1963)
G. annulata	T. annulata		

Table IV : Differentiating Features between T.lawrencei and T. parva.

Criterion	T. lawrencei	T. parva
Average macroschizont size	5 µm	8 µm
Parasitized lymphoid cells	5%	60%
Microschizonts and piroplasms	rare	Numerous

Table V : A summary of information on buffaloes used in experiments

Buffalo Number	Sex	Site of capture	Place of captivity
W 105-75	Female	Mara, Kenya.	Veterinary Department Research Laboratories Kabete, Kenya (VDRLK)
W 113-75	Female	" "	VDRLK, Kenya.
W 212-75	Male	Timau, Kenya.	VDRLK, Kenya.
W 120-76	Male	Rumuruti, Kenya.	VDRLK, Kenya.
W 121-76	Female	" "	VDRLK, Kenya.
W 122-76	Female	" "	VDRLK, Kenya.
W 325-76	Female	" "	VDRLK, Kenya.
WS 189-77	Male	*Born at Whipsnade in 1976	Zoological Society of London, Whipsnade Park.

* Born to a female which was also born at Whipsnade Park

Table VI : Status and origin of non-infected cattle used in experiments

Animal Number	Age	Sex	Breed	Tissue from which lymphoid cells were prepared	Place where animal was kept
-	8 month foetus	Male	Zebu	Spleen	Kenya Meat Commission (KMC) Athi River Abattoir
-	6 month foetus	Female	Friesian	Spleen	KMC, Athi River Abattoir
-	One week old calf	Male	Jersey	Blood and lymph node	Central Veterinary Laboratories (CVL), Weybridge, Surrey.
C74/77	Steer	Male	Friesian	Blood	CVL, Weybridge, Surrey.
608	Adult	Female	Friesian	Blood	Royal Veterinary College (RVC), Fieldstation, Potter's Bar.
745	Adult	Female	Friesian	Blood	RVC, Fieldstation, Potter's Bar.

Table VII:

Stabilates of Theileria infective particles inoculated into cattle for either immunization or for further stabilate preparations

Stabilate Number	Parasite	Other Information
Stabilate 10	T. parva (Muguga)	Brocklesby et al. (1961)
Stabilate 92	T. parva (Muguga)	A mixture of these constituted the "Stabilate cocktail" (Radley et al., 1975a)
Stabilate 102	T. parva (Kiambu)	
Stabilate 112	T. lawrencei (Serengeti transformed)	
Stabilate 128	T. lawrencei	Prepare from infected ticks from the vegetation in the buffalo paddock (Young et al., 1977a).

Table VIII:A summary of the history of cattle

Animal Number	Breed and sex	Immunized with	Duration of exposure in the paddock	Macro-schizonts seen
L 62	Ayshire steer	Cocktail of stabilates 4 months before exposure in paddock	7 days	9-29 days post exposure in the paddock
L 81	Friesian heifer	Stabilate 128, 32 days before exposure	7 days	6-26 days post exposure
L 89	Ayrshire steer	Stabilate 128, 32 days before exposure	7 days	6-38 days post exposure
L 493	Ayrshire steer	Not immunized but inoculated with stabilate 10 for purposes of preparation of further stabilates	Not exposed	Day 9 post inocula-tion up to the time of death

used for isolation of cell lines

Fever	Time to death	Time tissue culture samples were collected
Did not develop fever	35 days post exposure	Within an hour following death
Did not develop fever	Recovered	23 days post exposure
Had a fever between 16 and 19 days post exposure	39 days post exposure	20 days post exposure
Had a fever from day 12 up to the time of death	22 days post inoculation	Within an hour following death

TABLE IX A Summary of Various Theileria-infected Cell Lines Used in Experiments.

Theileria species	Cell Line	Isolated from	Isolated by	Passed on to	Obtained by the author from	Other Information
T. lawrencei	L62	Ayrshire steer	The author of this thesis	-	-	Available in Chapter 4
T. lawrencei	L81	Friesian heifer	The author	-	-	Available in Chapter 4
T. lawrencei	L89	Ayrshire steer	The author	-	-	Available in Chapter 4
T. lawrencei	L325	African buffalo	The author	-	-	Available in Chapter 4
T. parva (Muguga)	L493	Ayrshire steer	The author	-	-	Available in Chapter 4
T. parva	E174	Ayrshire steer	Dr. C.G.D. Brown at EAVRO, Muguga)	Wellcome Research Laboratories (WRL) Beckenham, Kent	Dr. N. McHardy (WRL, Beckenham)	Cell line isolated in Sept 1971 and passed on to WRL at the twelfth passage. Received by author at passage 250.
T. annwata	S3	Friesian steer	Razi Institute,* Iran	Centre for Tropical Veterinary Medicine (CTVM), Edinburgh.	Dr. C.G.D. Brown, CTVM, Edinburgh.	Parasite described as very virulent but does not produce piroplasms
Tannulata	S15	Friesian steer	Razi Institute,* Iran	CTVM, Edinburgh	Dr. C.G.D. Brown, CTVM, Edinburgh	Parasite described as moderately virulent but does not produce piroplasm
Tannulata	S19/224	Friesian steer	Razi Institute,* (Iran as cell lineS19)	CTVM, Edinburgh	Dr. C.G.D. Brown, CTVM, Edinburgh.	Parasite described as very virulent and produces piroplasms. The cell line S 19 was passed through calf no. 224 at CTVM, Edinburgh.

*Hooshmand-Rad and Hashemi-Fesharki (1968)

TABLE IX A Summary of Various Theileria-infected Cell Li

Theileria species	Cell Line	Isolated from	Isolated by
T. lawrencei	L62	Ayrshire steer	The author of this thesis
T. lawrencei	L81	Friesian heifer	The author
T. lawrencei	L89	Ayrshire steer	The author
T. lawrencei	L325	African buffalo	The author
T. parva (Muguga)	L493	Ayrshire steer	The author
T. parva	E174	Ayrshire steer	Dr. C.G.D. Brown at EAVRO, Muguga)
T. annwata	S3	Friesian steer	Razi Institute,* Iran
Tannulata	S15	Friesian steer	Razi Institute,* Iran
Tannulata	S19/224	Friesian steer	Razi Institute,* (Iran as cell lineS19)

*Hooshmand-Rad and Hashemi-Fesharki (1968)

ines Used in Experiments.

Passed on to	Obtained by the author from	Other Information
-	-	Available in Chapter 4
-	-	Available in Chapter 4
-	-	Available in Chapter 4
-	-	Available in Chapter 4
-	-	Available in Chapter 4
Wellcome Research Laboratories (WRL) Beckenham, Kent	Dr. N. McIlardy (WRL, Beckenham)	Cell line isolated in Sept 1971 and passed on to WRL at the twelfth passage. Received by author at passage 250.
Centre for Tropical Veterinary Medicine (CTVM), Edinburgh.	Dr. C.G.D. Brown, CTVM, Edinburgh.	Parasite described as very virulent but does not produce piroplasms
CTVM, Edinburgh	Dr. C.G.D. Brown, CTVM, Edinburgh	Parasite described as moderately virulent but does not produce piroplasm
CTVM, Edinburgh	Dr. C.G.D. Brown, CTVM, Edinburgh.	Parasite described as very virulent and produces piroplasms. The cell line S 19 was passed through calf no. 224 at CTVM, Edinburgh.

Table X: Lymphoblastoid cell lines not infected with Theileria

Cell line	From:	With:	Isolated by:	Passed on to:	Other Information
BL-3	Adolescent steer in California, U.S.A.	A fatal case of bovine lymphosarcoma	?	E.A.V.R.O., Muguga, by Dr. J. Weaver	Received from E.A.V.R.O., Muguga, in the U.K. by Dr. S.P. Morzaria C.V.L., Weybridge, Surrey.
20	Friesian female calf at C.V.L., Weybridge.	A fatal case of multicentric juvenile form of sporadic leukosis	Dr. S.P. Morzaria	—	Cell line derived from cultures of bronchial lymph nodes and the cells grew singly in culture
24	Friesian female calf at C.V.L., Weybridge.	Fatal case of multicentric juvenile form of sporadic leukosis	Dr. S.P. Morzaria	—	This cell line was derived from the same animal as cell line 20. The cultures were set up from axillary lymph node and cells grew in clumps

Table XI : Results of attempts to establish T. lawrencei-infected cattle cells in culture

Animal number	Tissue used and extent of parasitosis	Cultures set up: Culture number	(a) with BESC's	(b) without BESC's	Period to transformation (days)	Other Information
L 62	Spleen (Sch.+++)*	1		+	25	Discarded after transformation
		2		+	30	Used for pooling cells and cryopreservation
L 81	Blood (Sch.+)*	1		+	18	Used for pooling cells and cryopreservation
		2	+		-	Discarded before transformation due to fungal contamination
	Left parotid lymph node (Sch.++)*	3		+	-	
		4	+		-	
		5	+		30	Discarded after transformation

*Sch.+ - at least a schizont per 5 or more fields of light microscope examination (X1000)

Sch.++ - at least a schizont per 3-5 fields of light microscope examination (X1000)

Sch.+++ - at least a schizont seen in every field of light microscope examination (at X1000)

Table XI: continued

Animal number	Tissue used and extent of parasitosis	Culture number	Cultures set up: (a) with BESC's	Cultures set up: (b) without BESC's	Period to transformation (days)	Other Information
L 89	Blood (Sch.+)	1	+	Pooled after 14 days in culture	23	Used for pooling cells and cryopreservation
		2	+			
		3	+			
		4	+		31	Discarded after transformation
		5	+		31	
		6	+		27	
	Left parotid lymph node (Sch.+)	7		+	33	
		8	+		26	

*Sch.+ - at least a schizont per 5 or more fields of light microscope examination (X1000)

Sch.++ - at least a schizont per 3-5 fields of light microscope examination (X1000)

Sch.+++ - at least a schizont seen in every field of light microscope examination (at X1000)

Table XII: Theileria piroplasms, serology and the distribution of harvested mononuclear cells from T. lawrencei-infected buffaloes

Buffalo number	Theileria piroplasms per 1000 erythrocytes	IFA antibody titres to T. parva schizont antigen	Leukocyte counts per millilitres ($x10^3$)	Total mononuclear cells harvested ($x10^7$)	Distribution of mononuclear cells in: (a) cultures with BESC's ($x10^7$)	(b) cultures without BESC's ($x10^7$)
W 105-75	9	< 40	7.1	8.7	3.5	5.2
W 113-75	5	< 40	7.4	9.8	3.9	5.9
W 212-75	< 1	40	4.4	5.0	2.0	3.0
W 120-76	< 1	< 40	5.2	4.0	4.0	
W 121-76	< 1	< 40	6.9	10	4.0	6.0
W 122-76	< 1	< 40	3.7	7.7	3.0	4.7
W 325-76	< 1	< 40	7.1	6.0	2.4	3.6*

* The only culture where successful isolation was made

Table XIII: Numbers, viability and successes of attempts to resuscitate cryopreserved cells

Theileria species	Cell line	Total cells harvested ($\times 10^8$)	Cells per cryoampoule ($\times 10^7$)	% cells excluding the dye	Resusitation: (a) 24 hours after storage in liquid Nitrogen (i) without BESCs: Number of successes / Number of trials	(a) (ii) with BESCs: Number of successes / Number of trials	(b) After arrival into the U.K. (i) without BESCs: Number of successes / Number of trials	(b) (ii) with BESCs: Number of successes / Number of trials	Total Successes / Total trials
T. lawrencei	L 62	1.8	1.8	85	1/1	Not done	0/1	1/1	2/3
T. lawrencei	L 81	1.6	1.6	73	0/3	1/1	Not done	1/1	2/5
T. lawrencei	L 89	2.0	2.0	87	1/1	Not done	0/1	1/1	2/3
T. lawrencei	W 325	2.9	2.9	89	0/1	1/1	Not done	1/1	2/3
T. parva (Muguga)	L 493	2.3	2.3	90	1/1	Not done	0/1	1/1	2/3

Table XIV : The percentage of cells parasitized at various passages

Cell line	Passage number							
	9	18	30	38	49	60	69	81
L 62	93.0	96.8	91.0	98.2	94.0	96.4	97.6	96.2
L 81	95.6	98.0	96.4	96.8	98.0	94.8	-	-
L 89	92.8	96.2	94.0	92.2	97.0	95.0	98.2	93.4
W 325	89.2	95.0	94.2	96.8	95.4	93.8	97.0	96.2
L 493	96.6	93.8	95.2	94.8	95.0	99.2	96.4	94.6

Table XV : The percentage of cells in mitosis at various passages

Cell line	Passage number							
	9	18	30	38	49	60	69	81
L 62	4.2	2.8	5.2	3.8	2.0	4.6	2.4	4.0
L 81	1.8	3.4	2.6	4.0	5.4	3.6	-	-
L 89	2.0	4.8	3.0	6.2	2.4	3.8	4.2	3.4
W 325	5.0	2.2	3.8	2.0	4.4	3.0	2.4	4.8
L 493	3.0	3.8	1.6	2.4	4.8	3.4	1.8	2.8

Table XVI: The percentage of multinucleated cells at various passages

Cell line	Passage number							
	9	18	30	38	49	60	69	81
L 62	9.0	4.4	6.2	2.4	4.6	2.4	8.2	7.4
L 81	4.8	3.8	7.2	5.6	9.8	9.0	-	-
L 89	3.0	2.8	4.6	3.8	7.2	5.0	4.0	5.8
W 325	5.4	2.6	9.2	6.0	3.6	2.8	4.2	6.8
L 493	6.6	8.4	3.6	5.4	11.0	8.2	2.6	4.2

Table XVII: The mean schizont number (MSN) of each cell line at different passage levels

Cell line	Passage number															
	9		18		30		38		49		60		69		81	
		SD		SD		SD		SD		SD		SD		SD		SD
L 62	12.8	8.5	9.7	7.0	13.6	11.2	11.8	8.0	10.0	8.7	13.1	9.3	14.3	12.0	16.1	13.0
L 81	10.3	9.1	14.1	11.4	15.2	11.7	10.8	7.0	12.0	10.3	12.8	9.4	-		-	
L 89	13.6	11.5	11.2	9.0	15.7	13.2	9.2	9.0	12.7	10.7	10.8	8.0	13.0	11.6	12.0	10.9
W 325	5.8	3.7	4.5	4.0	7.1	5.2	5.2	4.8	5.9	3.4	8.1	7.3	4.8	3.8	6.3	3.3
L 493	14.7	12.0	12.2	9.5	10.4	9.3	14.0	11.8	13.5	12.5	10.8	8.6	12.7	10.4	10.5	8.0

SD = standard deviation

Table XVIII: Viability of cells used for preparation of lysates

Theileria species	Cell line	% Viable
T. lawrencei	L 62	86.0
T. lawrencei	L 81	79.0
T. lawrencei	L 89	92.0
T. lawrencei	W 325	89.0
T. parva	E 174	90.0
T. parva	L 493	90.0
T. annulata	815	88.0

Table XIX: The viability and uptake of tritiated thymidine by uninfected cattle lymphoid cells

Lymphoid cells from animal number	Percentage of cells excluding the dye:	Uptake of tritiated thymidine (Counts per minute) U* ($x10^3$)	C ($x10^3$)	SI	L ($x10^3$)	SI
745	83	5.72±234	20.87±1800	3.6	9.50±335	1.6
608	85	3.07±187	9.82±673	3.1	4.71±431	1.5
C74/77	89	3.77±246	11.30±2170	2.9	5.52±274	1.5
Calf+blood	77	6.34±281	31.82±2540	5.0	11.91±458	1.9
Calf lymph node	87	4.95±523	22.23±2630	4.5	6.97±739	1.4

U = Unstimulated cells

C = Con A stimulated cells

L = LPS stimulated cells

SI = Stimulation index

+ = No number

Table XX : Viability and characteristics of various lymphoblastoid cell lines

Theileria spp. in the cells	Cell line	Passage number	% viable cells	% parasitized cells	% multinucleate cells	% of cells in mitosis	Mean schizont nuclear number
T. lawrencei	L 62	30	95	99	3	3	10
"	L 81	30	80	98	4	4	15
"	L 89	30	87	96	5	2	11
"	W 325	30	94	97	3	5	5
T. parva	E 174	250	90	98	4	4	11
"	L 493	30	92	99	7	4	10
T. annulata	S 3	U*	89	96	5	6	10
"	S 15	U	93	98	6	3	13
"	S 19/224	30	90	95	5	2	9
	BL-3	30	94	-	-	-	-
uninfected cell lines	20	11	88	-	-	-	-
	24	12	91	-	-	-	-

U = Unknown

Table XXI : Protein content and enzyme activity of uninfected lymphoid cells

Animal number	Mitogenic stimulation	mg. of protein ml^{-1}	GPI: Units of enzyme activity ml^{-1}	GPI: Specific activity	ALD: Units of enzyme activity ml^{-1}	ALD: Specific activity
745	U*	6.90	2.20	0.32	0.39	0.05
	C*	8.30	2.85	0.34	1.04	0.12
	L*	11.20	0.55	0.05	0.43	0.04
608	U	5.85	1.20	0.21	-	-
	C	14.75	1.70	0.12	-	-
	L	7.50	0.75	0.10	-	-
C74/77	U	6.30	1.10	0.17	-	-
	C	10.40	1.80	0.17	-	-
	L	11.65	1.30	0.11	-	-
Calf blood	U	7.05	0.85	0.12	0.71	0.10
	C	8.90	1.35	0.15	1.10	0.14
	L	8.00	0.60	0.07	0.97	0.11
Calf lymph node	U	13.35	0.80	0.05	0.70	0.05
	C	12.70	1.50	0.12	1.25	0.09
	L	9.10	1.20	0.13	1.08	0.11

*U = Lysate from unstimulated cells

C = Lysates from Con A stimulated cells

L = Lysates from LPS stimulated cells

- = Not done

Table XXII: Protein content and enzyme activity for various lymphoblastoid cell lines

Cell line	Milligrams of protein ml-1	GPI: Enzyme units ml-	GPI: Specific activity	ALD: Enzyme units ml-1	ALD: Specific activity
L 62	11.25	5.70	0.50	8.90	0.79
L 81	8.10	3.60	0.44	5.40	0.67
L 89	23.20	9.25	0.39	10.40	0.45
W 325	13.70	9.30	0.67	10.40	0.76
L 493	12.10	6.85	0.56	6.30	0.52
E 174	24.65	11.35	0.46	9.75	0.39
S 3	7.50	7.15	0.95	3.90	0.51
S 15	10.75	4.30	0.40	7.30	0.67
S 19/224	14.00	9.30	0.66	8.40	0.69
BL-3	27.35	8.60	0.31	11.85	0.43
20	17.00	5.20	0.30	10.10	0.59
24	13.80	6.65	0.33	5.55	0.28

Appendix table 1

Preparation of "Lymphoprep" from 75% sodium metrizoate
(FAO Technical report 3, 1978)

Reagent	Volume (ml)	Procedure	Result
75%(W/v) sodium metrizoate solution	20	Mix thoroughly under sterile conditions	33.3%(W/v) sodium metrizoate solution
De-ionized distilled water	25		
33.3%(W/v) sodium metrizoate solution	30	Mix thoroughly under sterile conditions	Lymphoprep
8.0%(W/v) Ficoll solution	70		

Appendix table 2.

Preparation of GIBCO freeze-dried media

Preparation of 5 litres of medium "F 11"

All ingredients are added in the order shown below in a 5 litre container with a magnet bar inside, placed on a magnetic stirrer and stirred gently at room temperature throughout the procedure.

Ingredient	Volume (ml)	Weight (gm)	Units
Freeze-dried medium	—	48.00	—
Deionized distilled water	4500	—	—
Sodium bicarbonate	—	11.00	—
L-B-asparagine	—	0.50	—
Penicillin	—	—	500.000
Streptomycin	—	0.50	—
Kanamycin	—	0.50	—
IN Hydrochloric acid*	Add necessary volume to pH7.2	—	—
Deionized distilled water	Add appropriate volume to make up to 5000	—	—

*Dilute acid (1:10) before use and slowly (dropwise)
The medium is sterilized by filtration through 0.22 um pore size millipore membrane filter and the filtered medium is dispensed into appropriate containers with 1 ml samples from the first and last containers, and added to 2 separate nutrient broth samples (9 ml) for sterility test. Incubate the broth at 37° C for 96 to 120 hrs and check for sterility by making a smear and staining. Medium is then stored at + 4° C for a maximum of 4 weeks.

Preparation of medium "F 12"

The procedure is similar to that of "F 11" but only 1.75 gm of sodium bicarbonate is required. pH adjustment not necessary.

Preparation of medium "RPMI 1640"

The procedure is similar to that of "F 11" but only 10 gm of sodium carbonate is needed.

* Based on FAO technical report 3 (1978).

Appendix table 3. Preparation of trypsin solutions for trypsinization (FAO technical report 3, 1978)

Reagent	Weight (gm)	Volume (ml)	Resultant solution	Sterilization	Storage
NaCl	80.00	-			
K 1	4.00	-		By filtration	
Na_2HPO_4	0.60	-	Trypsin	through 0.22 um	+ 4° C
KH_2PO_4	0.60	-	diluent	pore sized	
Deionized distilled water	-	950.00	(10x)	membrane filters	
0.2% Phenol red	-	50.00			
Difcotrypsin	50.00	-	Trypsin	By filtration	
Glucose	25.00	-	solution	through 0.22 um	- 20° C
Trypsin diluent	-	500.00	(40x)	membrane filters	
Deionized distilled water	-	90.00	Normal		+ 4° C Not
Trypsin diluent (10x)	-	10.00	working		usually
Trypsin solution (40x)	-	2.50	strength	-	stored
Sodium bicarbonate 8.8%	-	1.00	trypsin		for
Penicillin	-	(500 units) 0.25	solution		more than
Streptomycin		500 ug 0.25	(1x)		2 weeks

Appendix Table 4

Enzyme assay reaction mixture for GPI (Kilgour, personal communication)

Reagent	Amount
0.3 M Tris/Cl buffer pH 8.0	2.000 ml
Distilled water	2.650 ml
NADP (10 mg ml^{-1})	0.600 ml
1 M $MgCl_2$	0.090 ml
Fructose-6-phosphate (10 mg ml^{-1})	0.600 ml
Glucose-6-phosphate dehydrogenase (100 units ml)	0.060 ml

Appendix Table 4

Enzyme assay reaction mixture for GPI (Kilgour, personal communication)

Reagent	Amount
0.3 M Tris/Cl buffer pH 8.0	2.000 ml
Distilled water	2.650 ml
NADP (10 mg ml^{-1})	0.600 ml
1 M $MgCl_2$	0.090 ml
Fructose-6-phosphate (10 mg ml^{-1})	0.600 ml
Glucose-6-phosphate dehydrogenase (100 units ml)	0.060 ml

Appendix Table 5

Enzyme assay reaction mixture for ALD (Based on Harris and Hopkinson, 1976)

Reagent	Amount
0.1 M Tris/Cl buffer pH 8.0	3.00 ml
Fructose 1,6-diphosphate (Na_2 Salt, $8H_2O$)	12.00 mg
NAD	2.4 mg
Sodium arsenate	7.2 mg
Glyceraldehyde-3-phosphate dehydrogenase (800 units ml^{-1})	6 µl
Distilled water	3.00 ml

Appendix table 6

Electrophoretic conditions

Enzyme	Tank Buffer	Gel buffer dilution	Voltage per cm	Time (min)	Authors
MDH	0.05M Na_2HPO_4 0.00TM citrate pH7.0	1:4	20 V	180	Gibson et al. 1978
ME	0.2M NaH_2PO_4 0.2M Na_2HPO_4 pH7.0	3:37	16 V	150	Miles et al. (1977)
G6PD	0.2M NaH_2PO_4 0.2M Na_2HPO_4 pH7.0	1:9	16 V	240	Al-Taqi and Evans (1978)
GAPDH	0.1M KH_2PO_4-Kolt; pH7.0	1:9	20 V	180	
ASAT	0.15M glycine-NaOH pH9.5	1:9	40 V	60	Kilgour and Godfrey (1973)
ALAT	0.15M Tris 0.0075M citric acid pH9.0	1:9	40 V	60	Kilgour and Godfrey 1973
PGM	0.1M Tris/0.1M Maleic acid 0.01M magnesium Chloride/0.01 EDTA; pH7	1:9	16 V	180	Miles et al. 1977
ALD	0.1M NaH_2PO_4 0.1M Na_2HPO_4 pH7.5	1:9	16 V	180	Modified by author of this thesis from Harris and Hopkins (1976)
GPI	0.2M NaH_2PO_4 0.2M Na_2HPO_4 pH7.8	3:37	16	180	Based on Miles et al.(1977) but pH modified by the author

Appendix table 7 Enzyme developers (mg and U

Enzyme	Developer buffer	Substrate	Coenzyme
MDH	0.1MTris-Hcl p 18.0	0.01MDL-Malic acid adjusted to pH7 with NaOH	0.30mg NAD
ME	0.1MTris-Hcl pH8.0	0.005MDL-Malic acid adjusted to pH7 with NaOH	0.20mg NADP
G6PD	0.1MTris-Hcl pH7.4	0.40mg D-glucose-6-phosphate (1Na)	0.40mg NADP
GAPDH	0.1MTris-Hcl pH7.4	10mg Fructose 1 6-diphosphate 2 aldolase (pre-incubation at 37°C) for 30 min	0.50mg NAD
ASAT	0.1MTris-Hcl pH7.4	2mg 2-oxo-glutarate 4mg aspartate	0.50mg NADH

as final concentrations ml^{-1})

Linking enzyme	Additional ions or other additions	Dye	Agar	Author(s)
—		0.50mg MTT 0.20mg PMS	6 mg	Gibson et al. (1978)
—	0.01$MgCl_2$	0.50mg MTT 0.20mg PMS	6 mg	Gibson et al. (1978)
—	0.45mg ATP	0.50mg MTT 0.20mg PMS	6 mg	Al-taqi and Evans (1978)
—	1mg sodium arsenate	0.50mg MTT 0.20mg PMS	6 mg	Gibson et al. (1978)
15 MDH (pig heart, 50% glycerol solution)	—	—	—	Kilgour and Godfrey (1973)

Appendix Table 7 cont..... Enzyme developers (mg and as final concentrations ml^{-1})

Enzyme	Developer buffer	Substrate	Coenzyme	Linking enzyme	Additional ions or other additions	Dye	Agar	Author(s)
ALAT	0.1MTris-Hcl pH7.4	2mg 2-oxo-glutarate 20mg alanine	0.50mg NADH	15 LDH (hog muscle, 50% glycerol solution)	—	—	—	Kilgour and Godfrey (1973)
PGM	0.1MTris-Hcl pH7.4	0.60mg glucose-1-phosphate with 1% glucose 1-6-diphosphate	0.40mg NADP	0.4 glucose-6-phosphate dehydrogenase	1.6mg $MgCl_2$	0.50mg MTT 0.20mg PMS	6 mg	Al-taqi and Evans (1978)
ALD	0.1MTris-Hcl pH7.4	0.20mg Fructose 1 6-diphosphate	0.40mg NAD	0.8 glyceral dehyde phosphate dehydrogenase	1mg sodium arsenate	0.50mg MTT 0.20mg PMS	6 mg	Harris and Hopkins (1976)
GPI	0.1MTris-Hcl pH8.0	0.40mg Fructose-6-phosphate	0.20mg NADP	0.4 glucose-6-phosphate dehydrogenase	0.01 M$MgCl_2$	0.50mg MTT 0.20mg PMS	6 mg	Gibson et al. (1978)

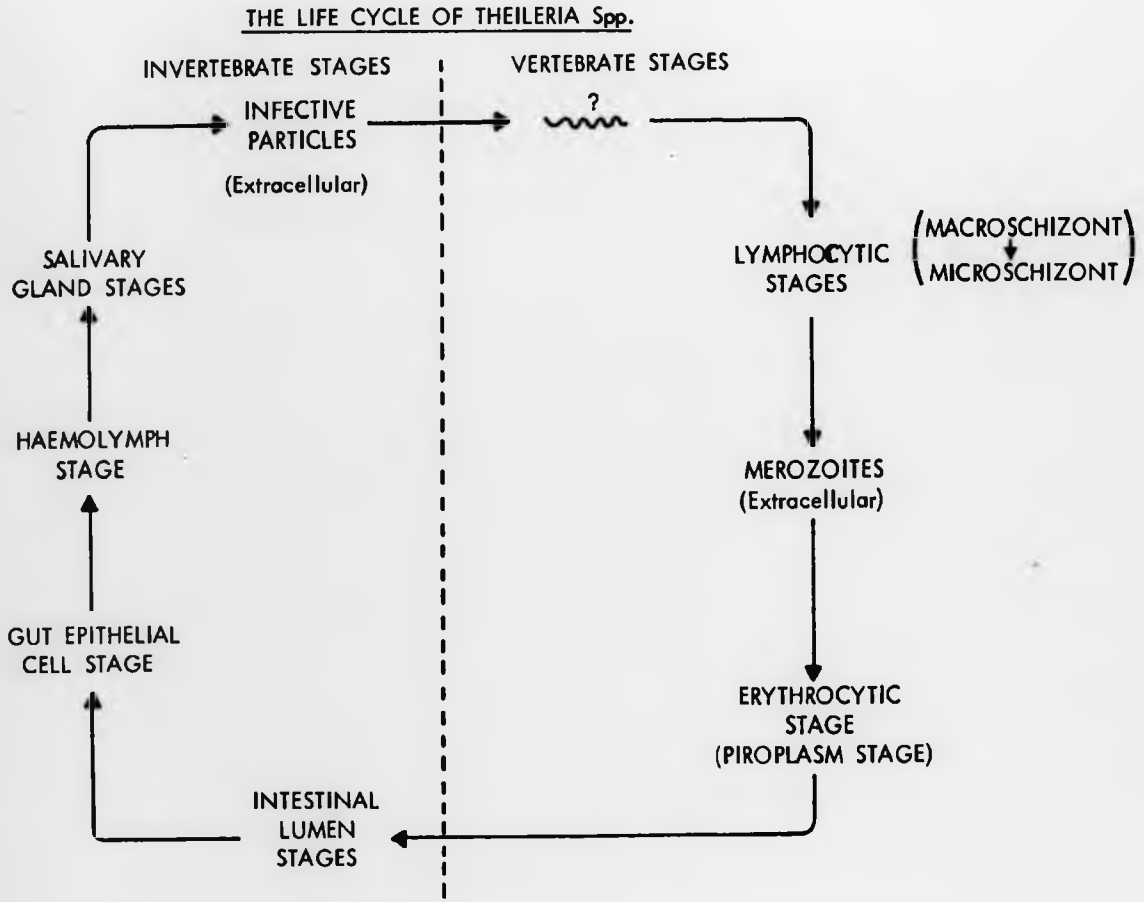

THE LIFE CYCLE OF THEILERIA Spp.
INVERTEBRATE STAGES
VERTEBRATE STAGES
INFECTIVE PARTICLES
(Extracellular)
?
SALIVARY GLAND STAGES
LYMPHOCYTIC STAGES
MACROSCHIZONT
MICROSCHIZONT
HAEMOLYMPH STAGE
MEROZOITES
(Extracellular)
GUT EPITHELIAL CELL STAGE
ERYTHROCYTIC STAGE
(PIROPLASM STAGE)
INTESTINAL LUMEN STAGES

Fig. 1

Figure 2.

Enzyme assay curves

The extreme right curve shows how time and absorbance units are read off the curve.

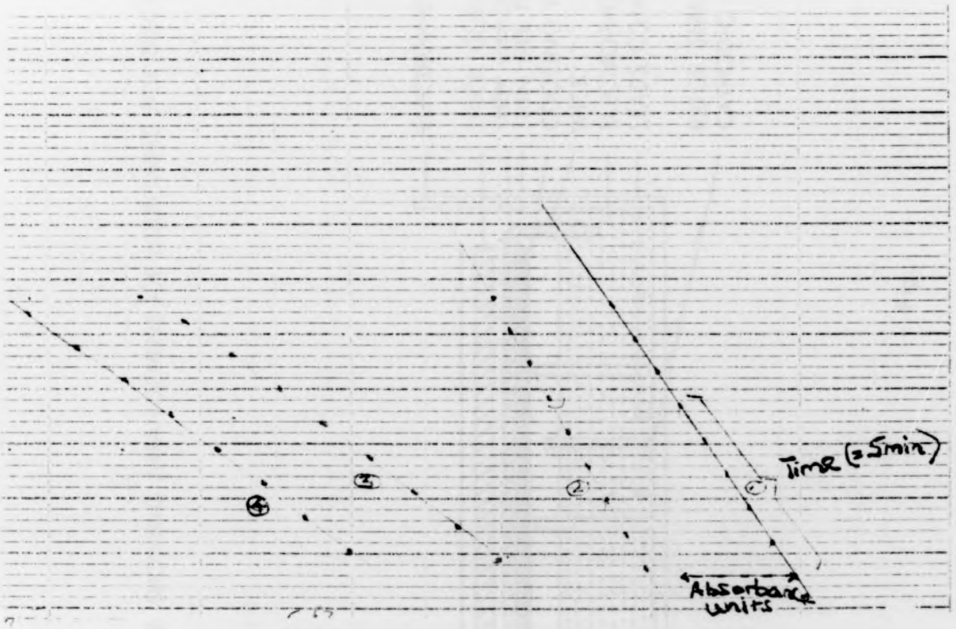


Fig. 2.

FIGURE SHOWING ARRANGEMENT OF EQUIPMENT FOR ELECTROPHORESIS

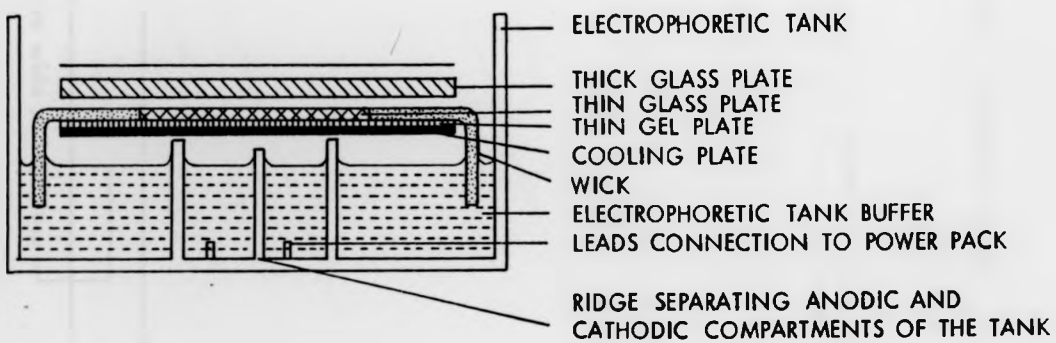


Fig. 3

DETAILS OF GEL - FORMING PLATE

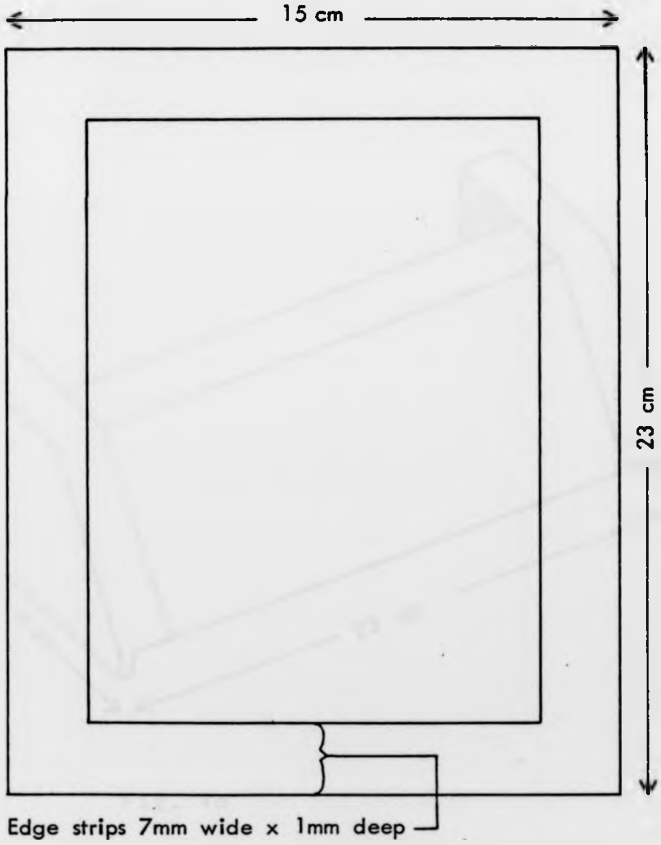


Fig. 4a

DETAILS OF GEL SPREADER

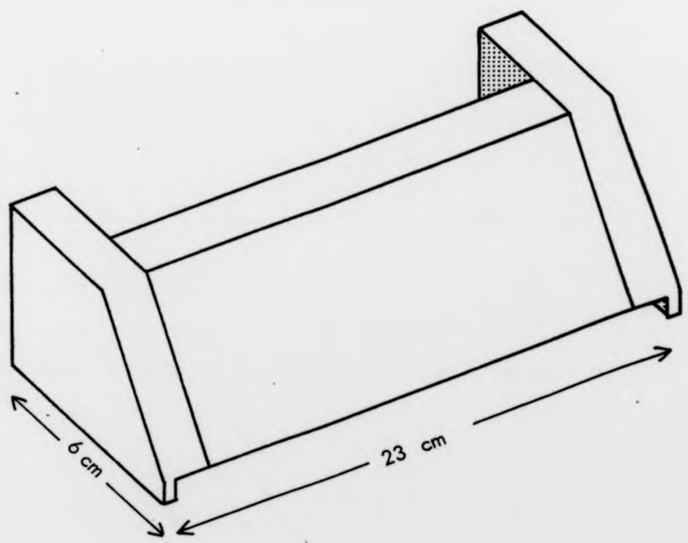


Fig. 4b

Key to Fig. 5


cooling curve for topmost ampoule


cooling curve for middle ampoule


cooling curve for bottom ampoule

COOLING CURVES FOR THEILERIA-INFECTED CELL SUSPENSIONS IN 1 ml GLASS AMPOULES IN A REVCO REFRIGERATOR

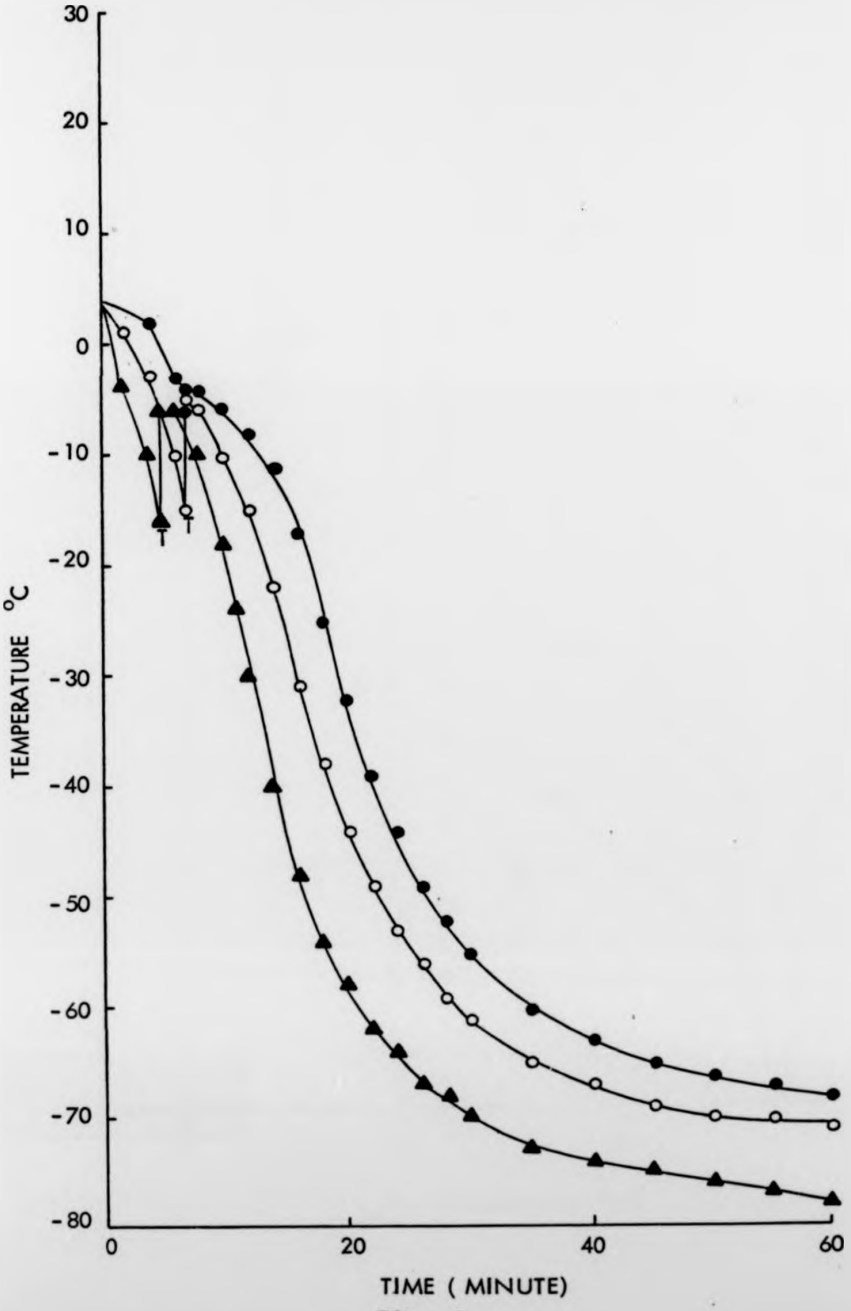


Fig. 5.

COOLING CURVES FOR THEILERIA-INFECTED CELL SUSPENSIONS IN 1 ml GLASS AMPOULES IN A REVCO REFRIGERATOR

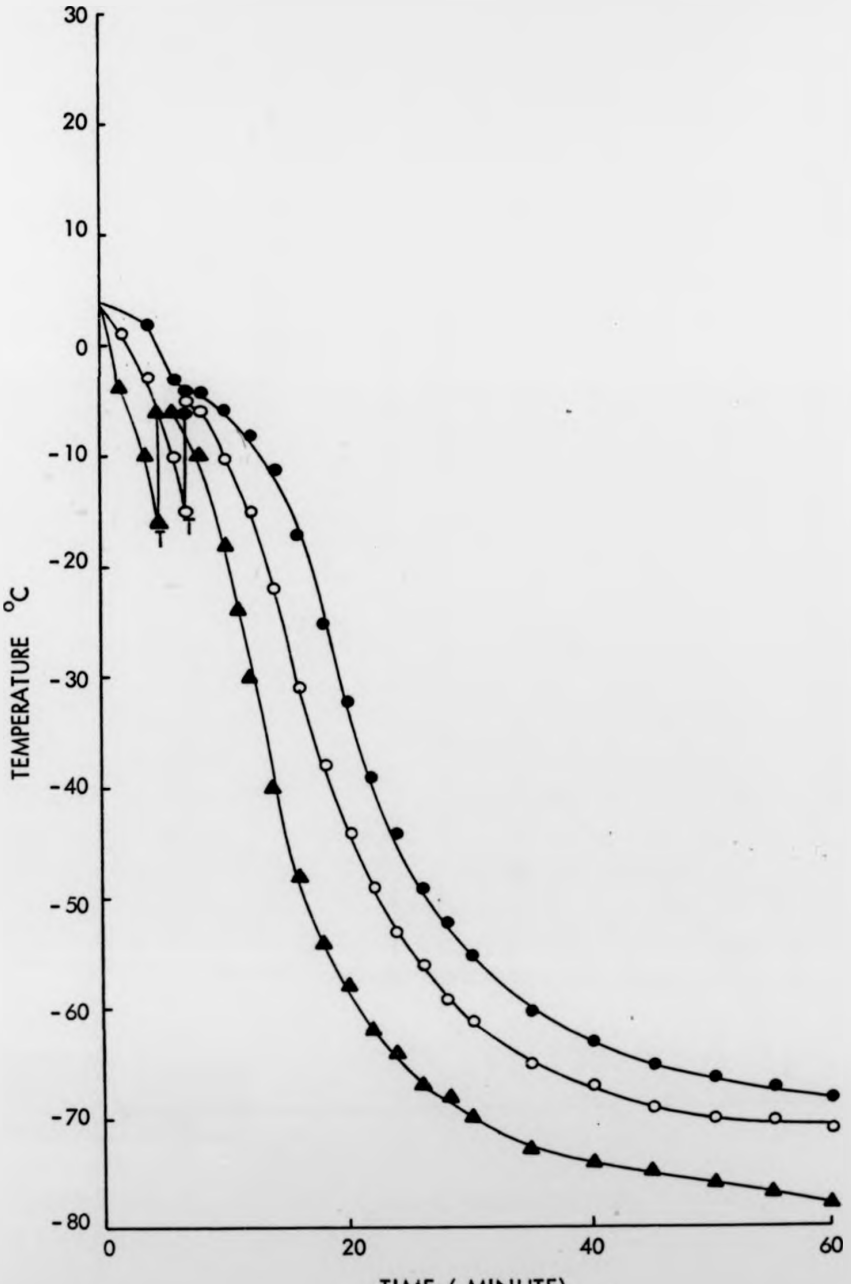


Fig. 5.

Key to Fig. 6

curve 'X' (cattle cells' growth curve)

curve 'Y' (buffalo cells' growth curve)

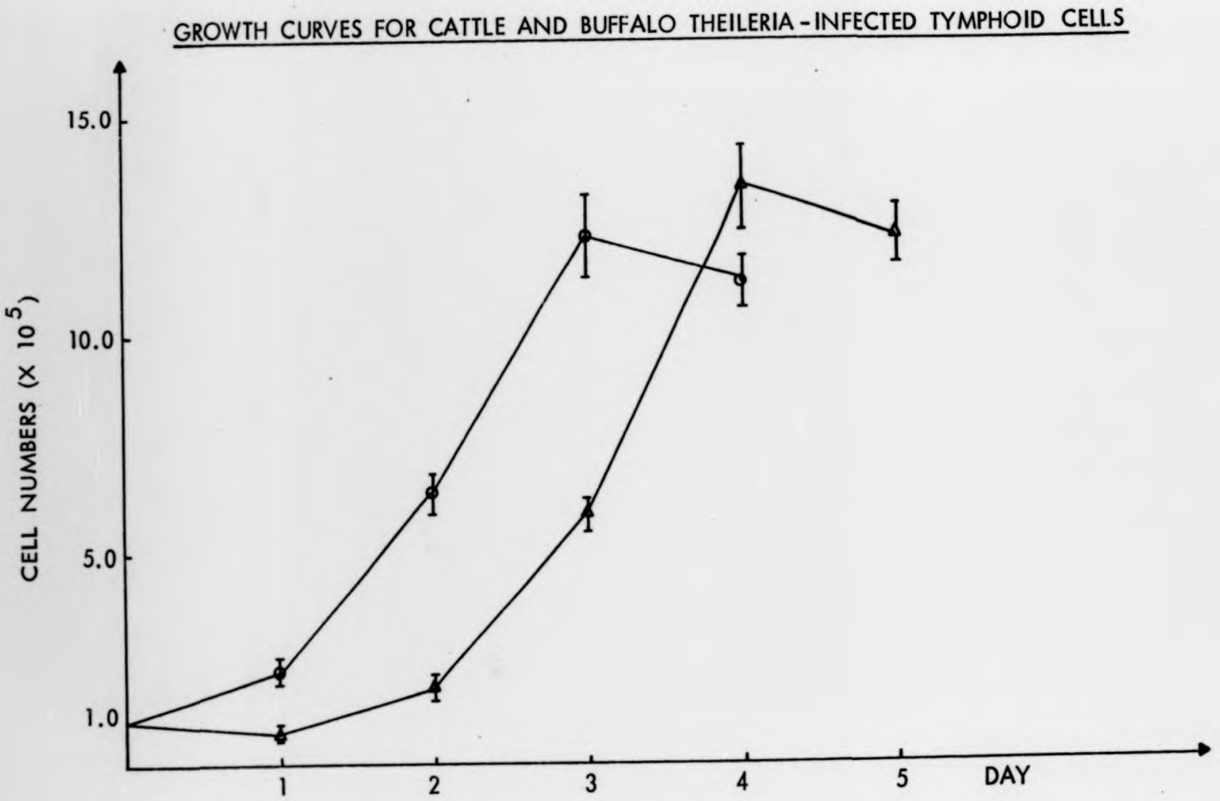

GROWTH CURVES FOR CATTLE AND BUFFALO THEILERIA - INFECTED TYMPHOID CELLS
CELL NUMBERS (X 10^5)
15.0
10.0
5.0
1.0
1
2
3
4
5
DAY

Fig. 6

Key to Fig. 7

T. lawrencei-infected lymphoblasts (cell line L 89); a smear from a 48-hour cell culture stained with Giemsa.

P = parasite

N = nucleus of host cell

Fig. 8

T. lawrencei-infected cattle lymphoblasts; smear from 48 hour cell culture stained with Giemsa. Note the two lymphoblasts in mitosis; the parasite (P) is in close association with host cell nuclear material (Nm).

Key to Fig. 7

T. lawrencei-infected lymphoblasts (cell line L 89); a smear from a 48-hour cell culture stained with Giemsa.

P = parasite

N = nucleus of host cell

Fig. 8

T. lawrencei-infected cattle lymphoblasts; smear from 48 hour cell culture stained with Giemsa. Note the two lymphoblasts in mitosis; the parasite (P) is in close association with host cell nuclear material (Nm).

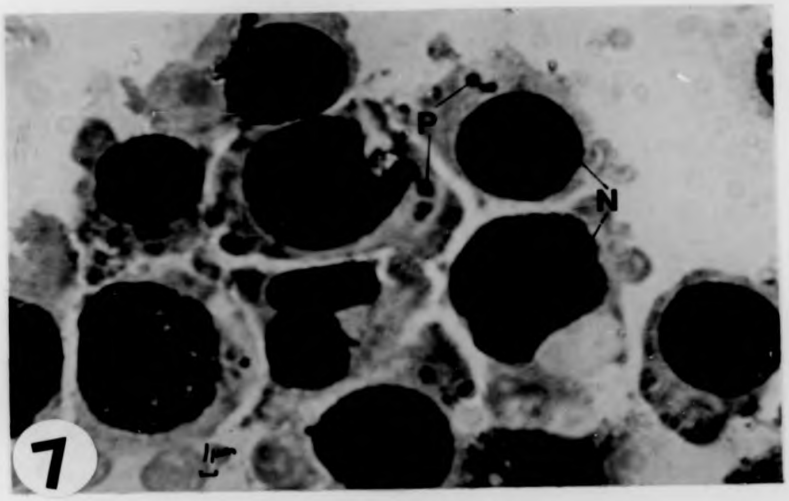
89);

th

iemsa.

asite

lear



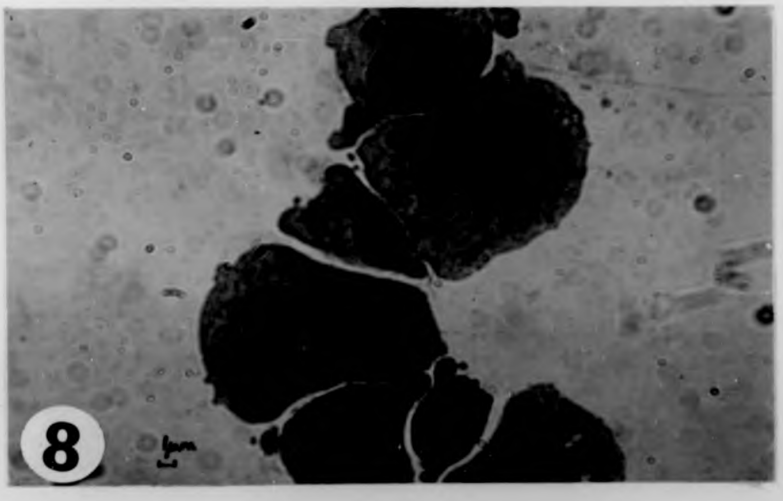
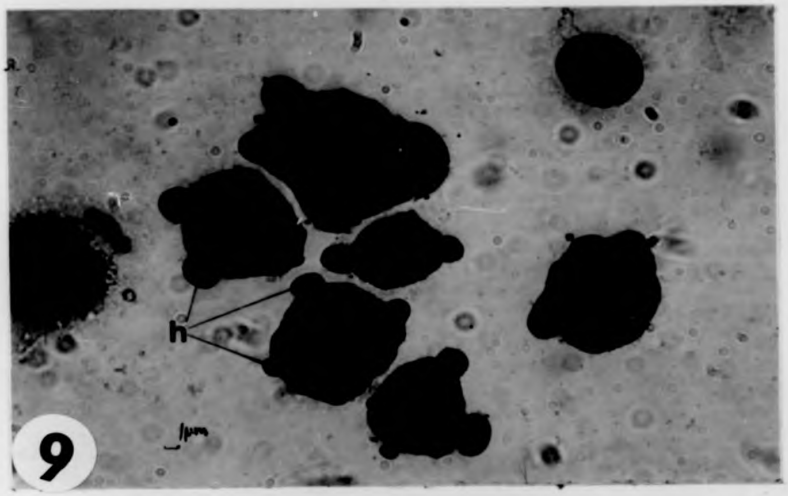


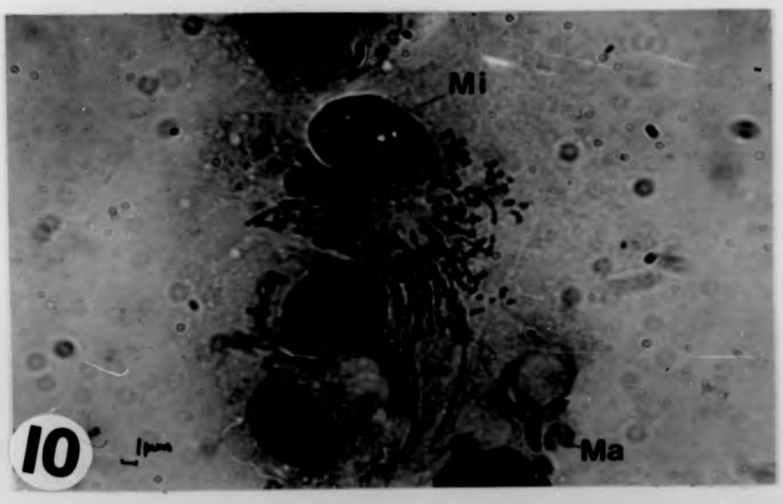
Fig. 9

T. lawrencei-infected buffalo lymphoid cells (cell line W 325) from a 48 hour cell culture, stained with Giemsa. Note the cytoplasmic herniations (h) on all cells.

Fig. 10

T. parva-infected lymphoblasts (cell line L 493); a smear from a 72 hour cell culture stained with Giemsa. Note the ruptured microschizont (Mi) and the extracellular macroschizont (Ma).





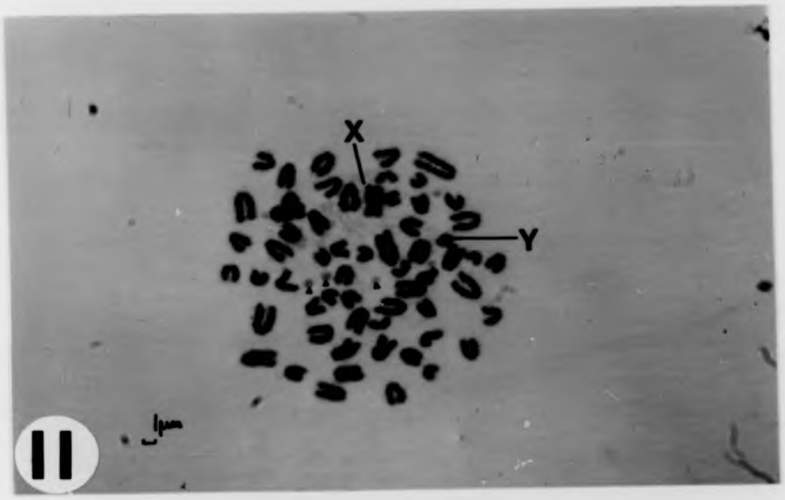
93);
ith
and

Fig. 11

Karyotype of T. parva-infected cell from a lymphoblastoid cell line (L 493) from spleen lymphoid cells of an Ayrshire steer. Note the two sex chromosomes X and Y, and the Theileria nuclei indicated by short arrows.

Fig. 12

Karyotype of T. lawrencei-infected cell from a lymphoblastoid cell line (L 81) from leukocytes of a Friesian heifer. The two X chromosomes and the marker chromosome (Mr) are clearly visible in the figure.


X
Y
11
1μm

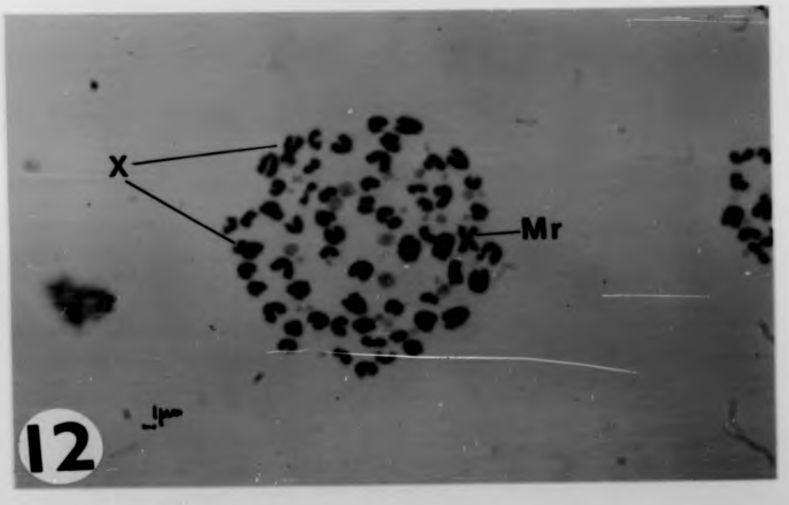
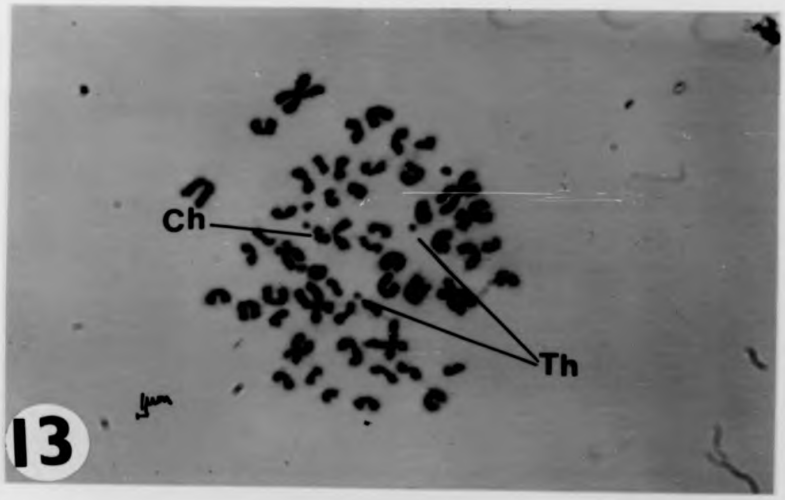

X
Mr
12
1μm

Fig. 13

Karyotype of an infected cell from a lymphoblastoid cell line (W 325) established from buffalo leukocytes. Note the various Theileria nuclei (Th) and one of the sex chromosome (Ch).


Ch
Th
13

Key to Fig. 14 - 36 c.

Al	Annulate lamella
C	Centriole
Ch	Chromosome
Cp	Cytoplasmic processes
G	Golgi apparatus
L	Lysosome
M	Mitochondria
Me	Merozoite
Mn	Microneme-like structures
Ms	Mitotic spindle
Mt	Microtubule
N	Nucleus
Nu	Nucleolus
P	Parasite
Pc	Parasite centriole
Plv	Parasite large vacuoles
Pn	Parasite nucleus
Pr	Polar ring
Psv	Parasite small vacuoles
R	Rhoptry-like bodies
S	Satellite bodies
Sc	Surface coat
St	Subpellicular tubules
T	Tubules
V	Vacuoles

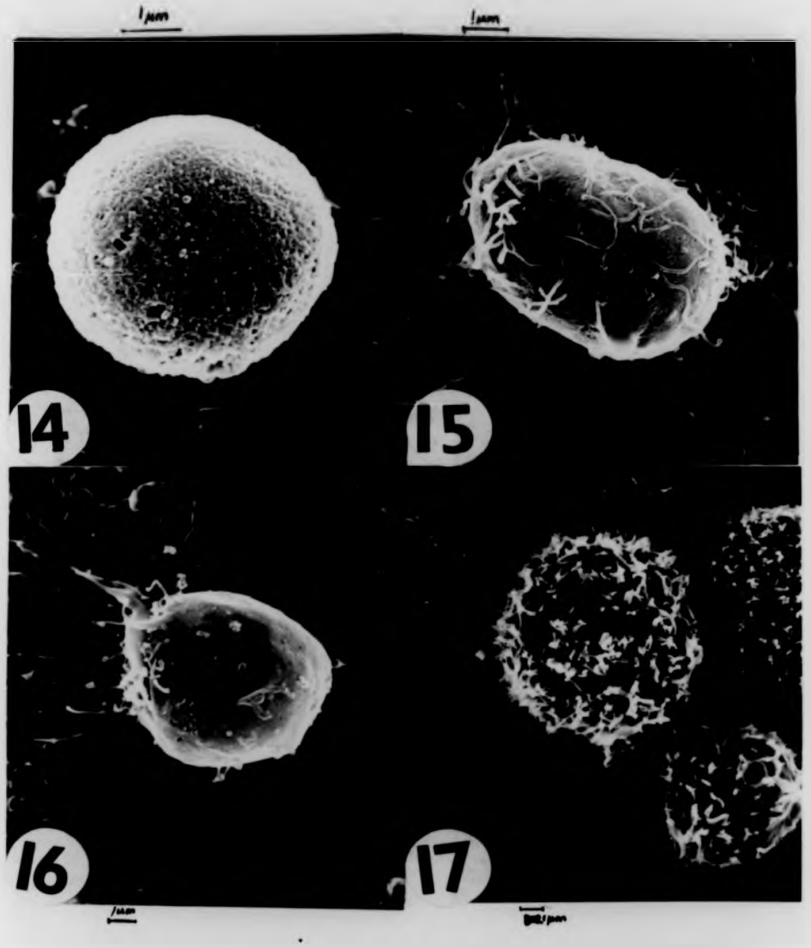


Fig. 14: A scanning electron micrograph of a lymphoid cell from blood of clinically normal cattle.

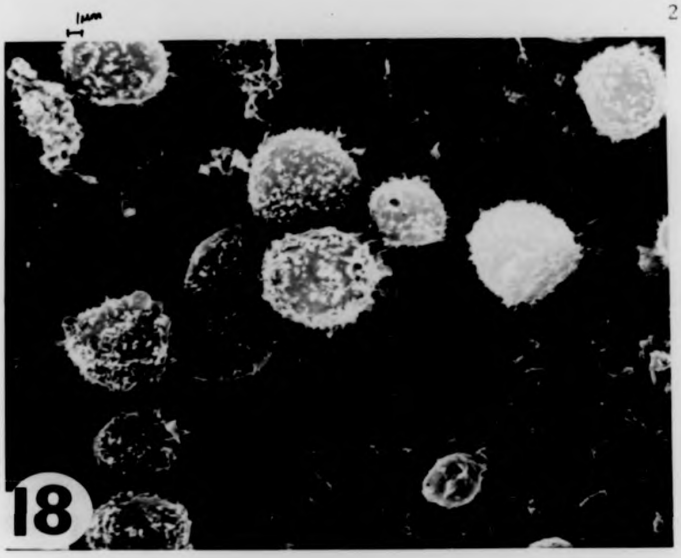
Fig. 15: A scanning electron micrograph of a T. parva-infected lymphoblastoid cattle cell from culture.

Fig. 16: A scanning electron micrograph of a T. lawrencei-infected lymphoblastoid cattle cell from culture.

Fig. 17: A scanning electron micrograph of a T. annulata-infected lymphoblastoid cattle cell from culture.

Fig. 18: A scanning electron micrograph of T. annulata-infected lymphoblastoid cattle cells demonstrating the variation in size of the cells.

Fig. 19: A stereoscopic pair of a scanning electron micrograph of T. parva-infected lymphoblastoid cattle cell showing how the cytoplasmic processes stand away from the cell surface.


1μm
18

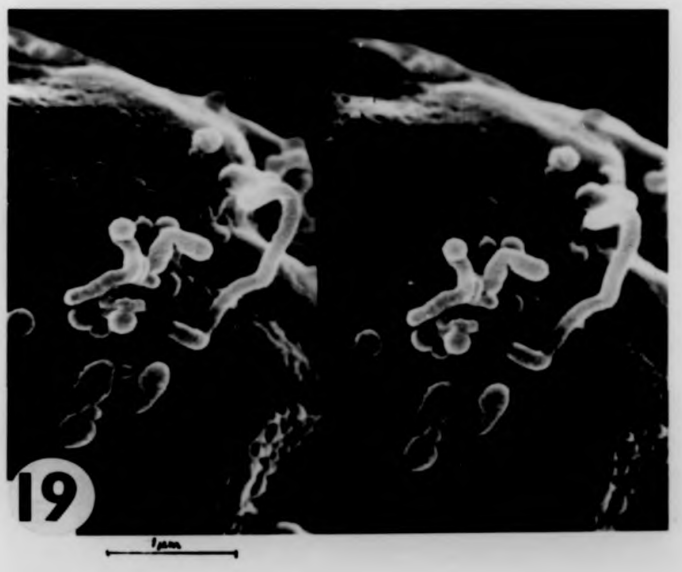

19
1μm

Fig. 20: A scanning electron micrograph of the surface of a lymphoid cell from a clinically normal cattle at a magnification of x80,000.

Fig. 21: A scanning electron micrograph of the surface of a T. parva-lymphoblastoid cattle cell from culture at x80,000 contrast the surface appearance with that of Fig. 20.

Fig. 22: A scanning election micrograph of the surface of a T. parva-infected lymphoblastoid cattle cell from culture showing the base of cytoplasmic processes.

face
al

face
from

face
tle
o-

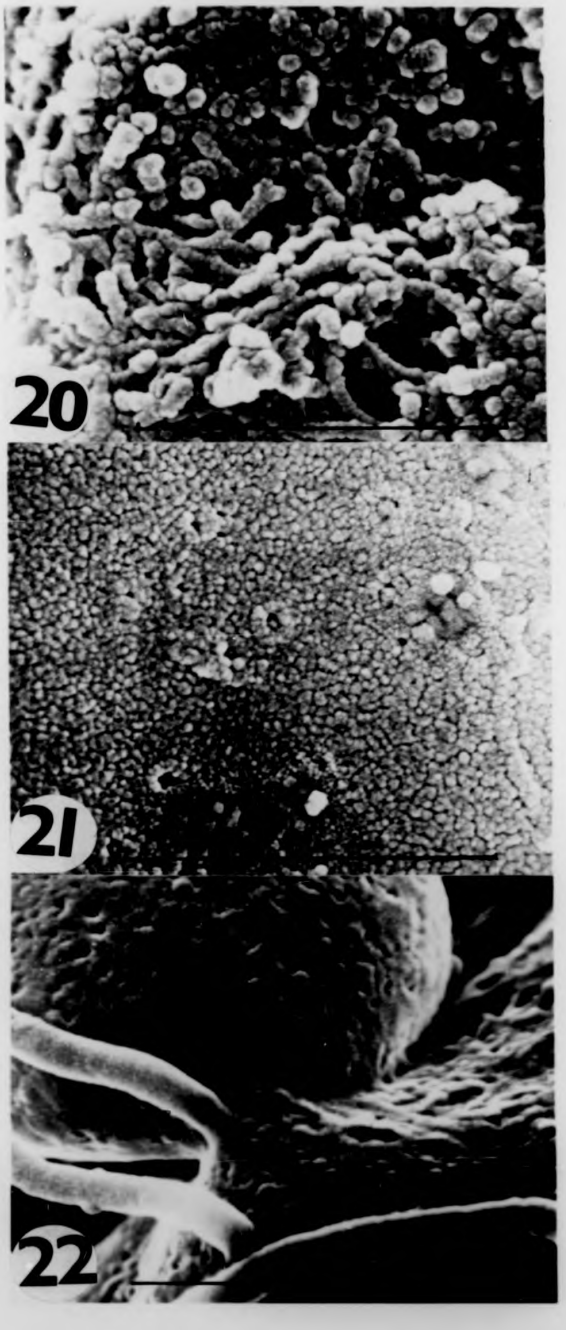


Fig. 23: A scanning electron micrograph of a T. annulata-infected lymphoblastoid cattle cell showing the bulbous termination of cytoplasmic processes.

Fig. 24: A scanning electron micrograph of a T. lawrencei-infected lymphoblastoid cattle cell showing bulbous termination of cytoplasmic processes.

Fig. 25: A scanning electron micrograph of a T. parva-infected lymphoblastoid cattle cell showing "herniation".

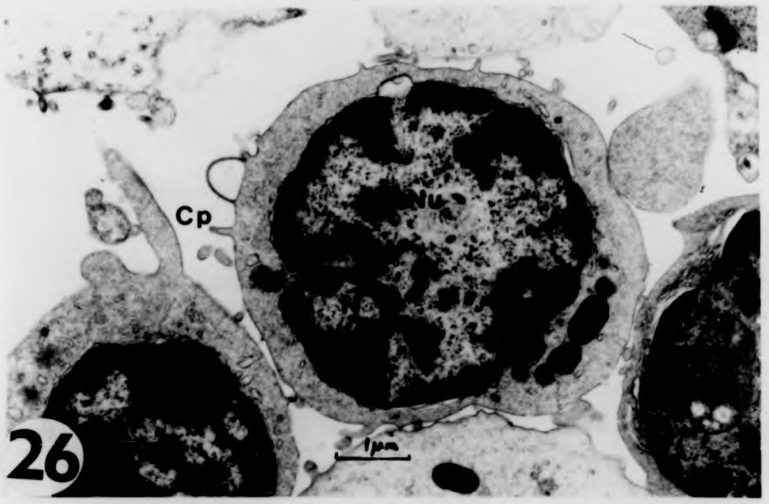
nulata-
ng the
ses.

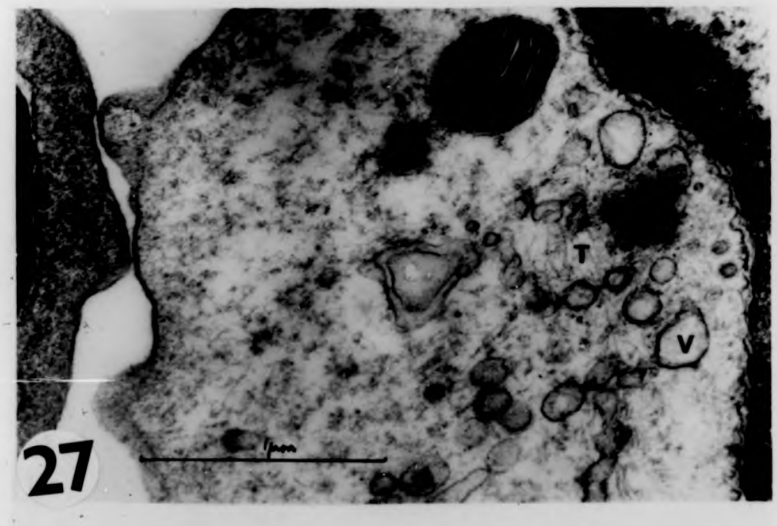
wrencei-
ng
ses.

rva-
ng

Fig. 26: An electron micrograph of lymphoid cells from blood of clinically normal cattle.

Fig. 27: A micrograph of a lymphoid cell from blood of a clinically normal cattle. Note the arrangement of cytoplasmic microtubules.


Cp
Nu
1µm
26


T
V
1µm
27

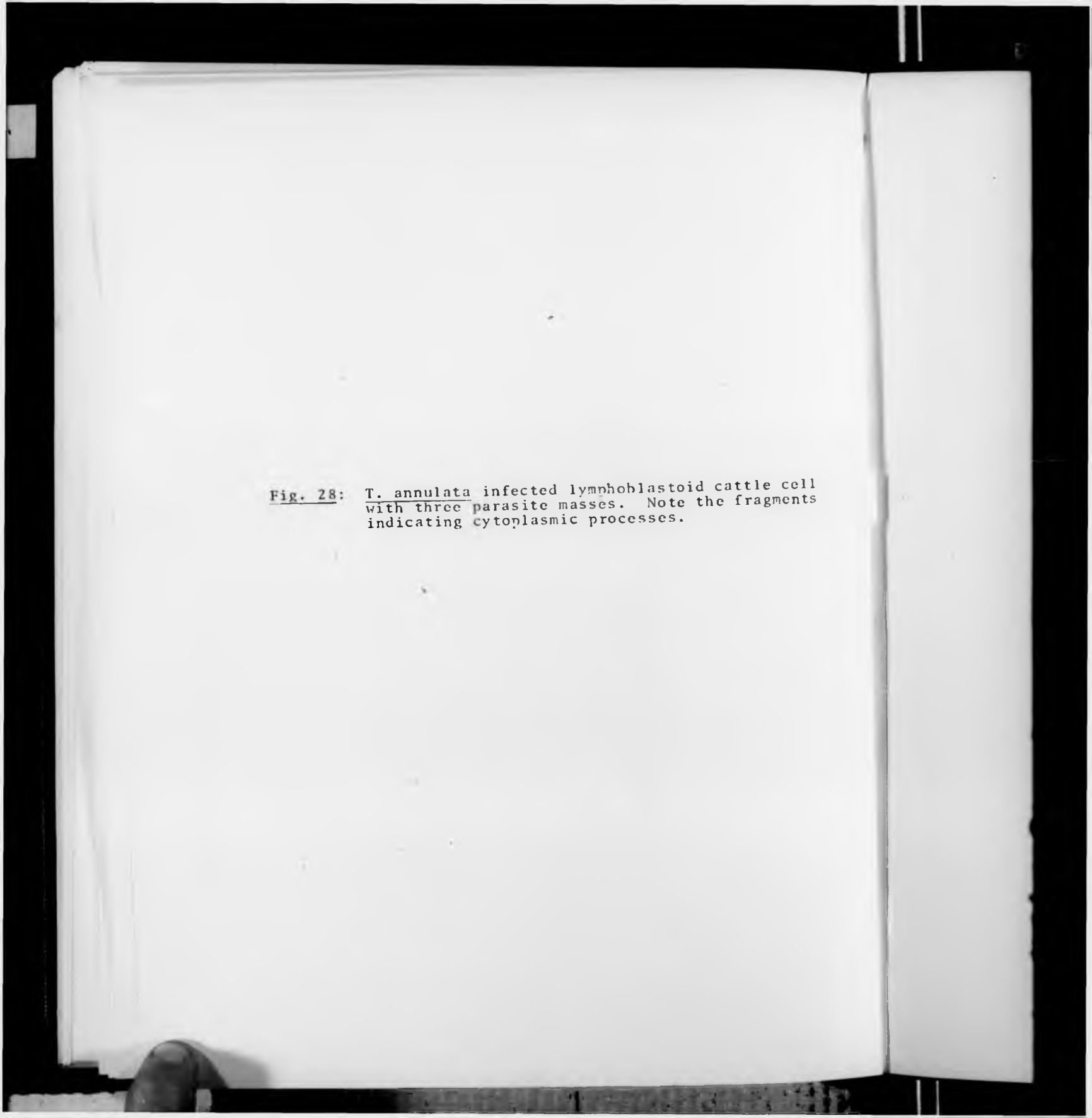
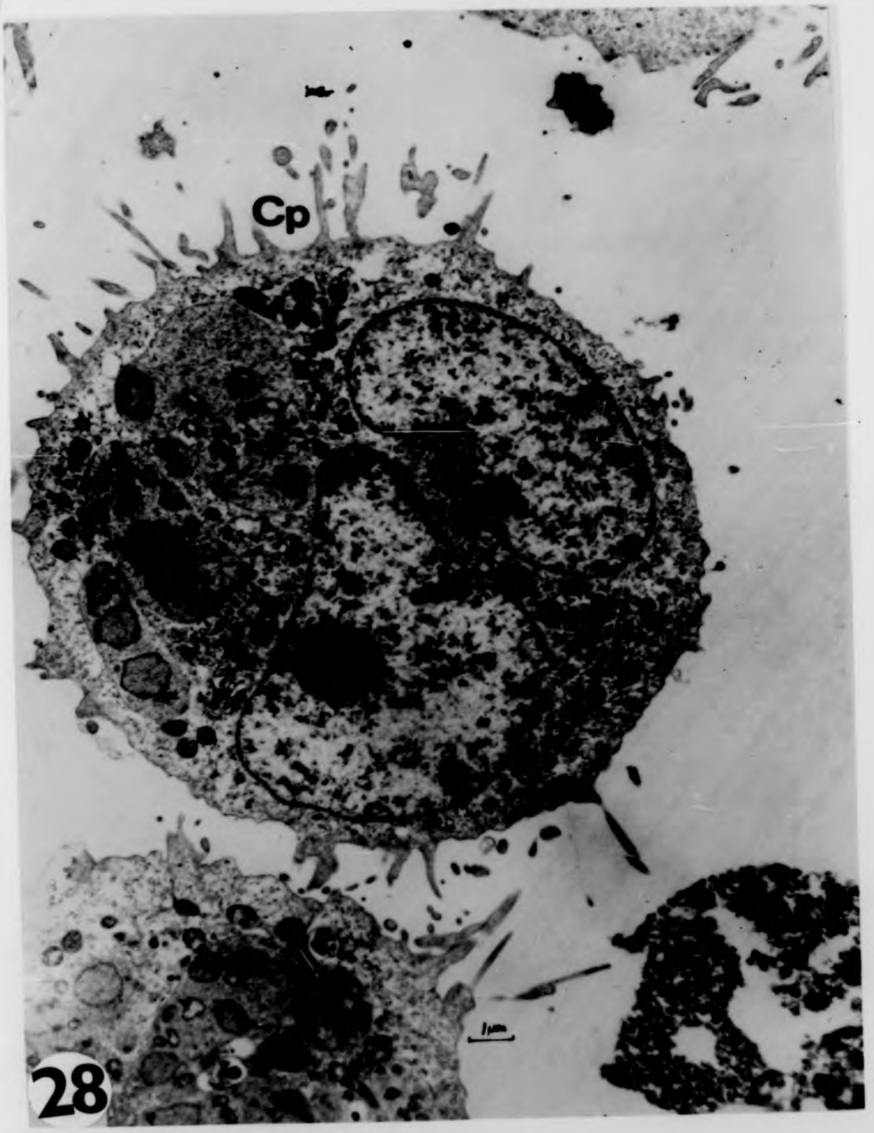


Fig. 28: T. annulata infected lymphoblastoid cattle cell with three parasite masses. Note the fragments indicating cytoplasmic processes.



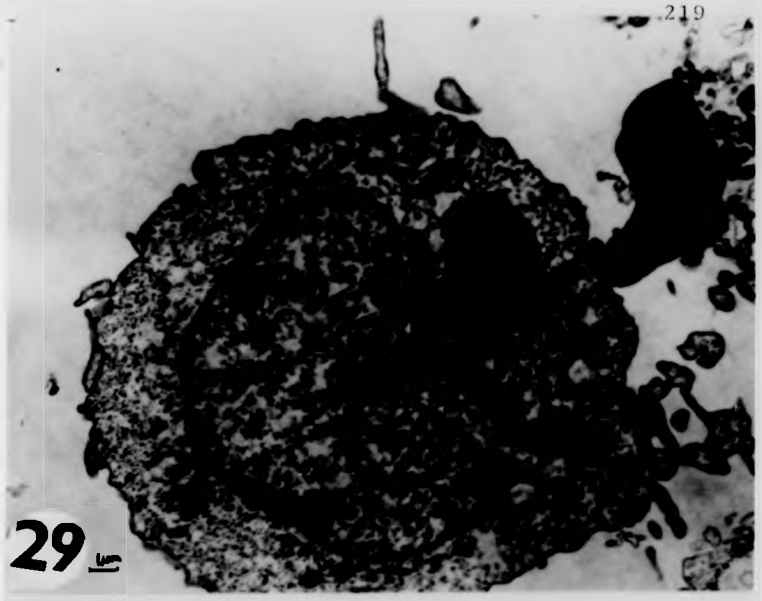
cell
ments

Fig. 29: T. parva-infected lymphoblastoid cattle cell with an active Golgi complex and a single layer annulate lamella.

Fig. 30a: A micrograph showing very active Golgi complex and annulate lamellae in two planes in another T. parva-infected lymphoblastoid cattle cell.

ell with

omplex
nother
cell.



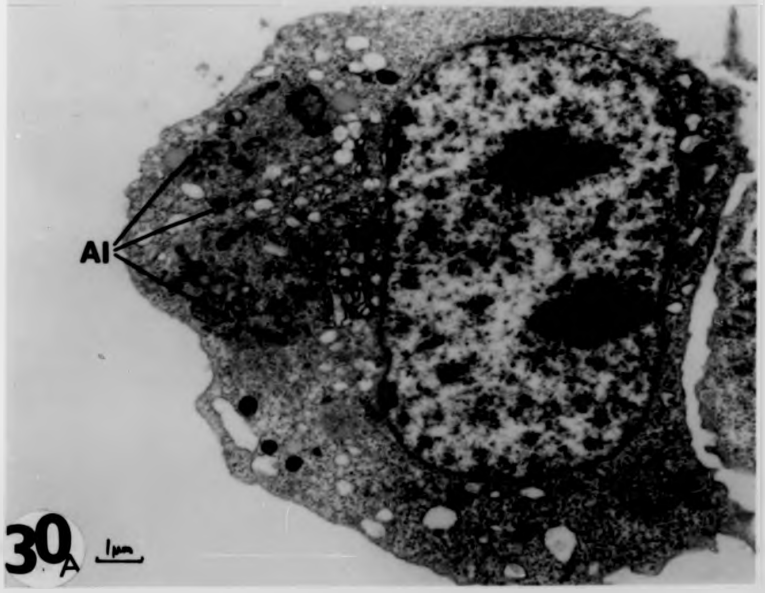
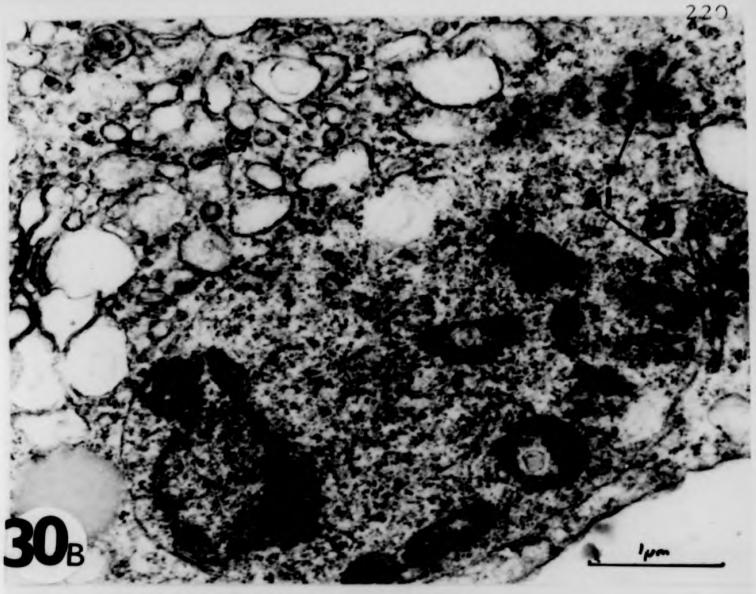


Fig. 30b: A micrograph showing the details of annulate lamellae and the apparent continuity of parasite and lymphoblast cytoplasm.

Fig. 31: An electron micrograph demonstrating single layer annulate lamella lying between two parasite masses. Note also the various parasite vacuoles.


30B
1μm

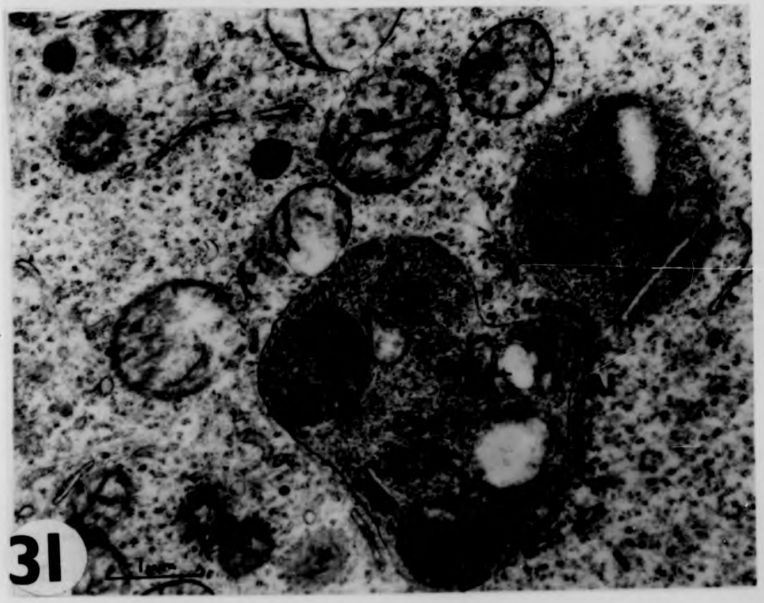

31

Fig. 32a: A T. annulata-infected lymphoblastoid cattle cell in mitosis. Note the marked vacirolation.

attle cell
on.

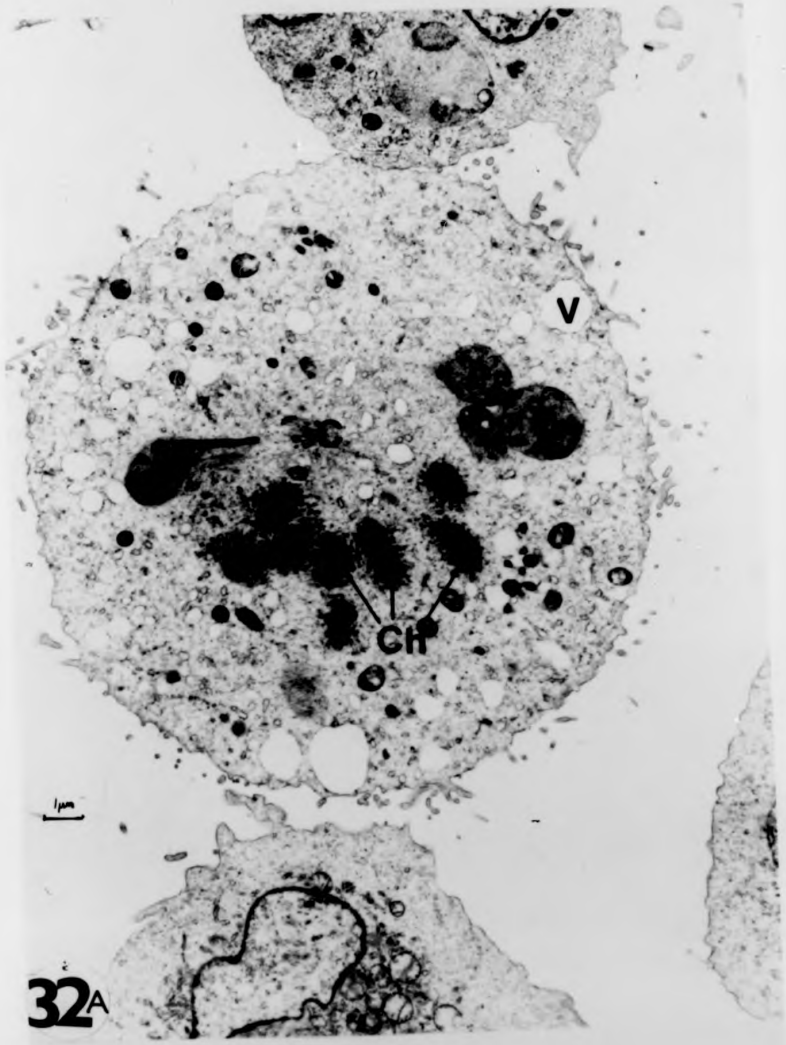
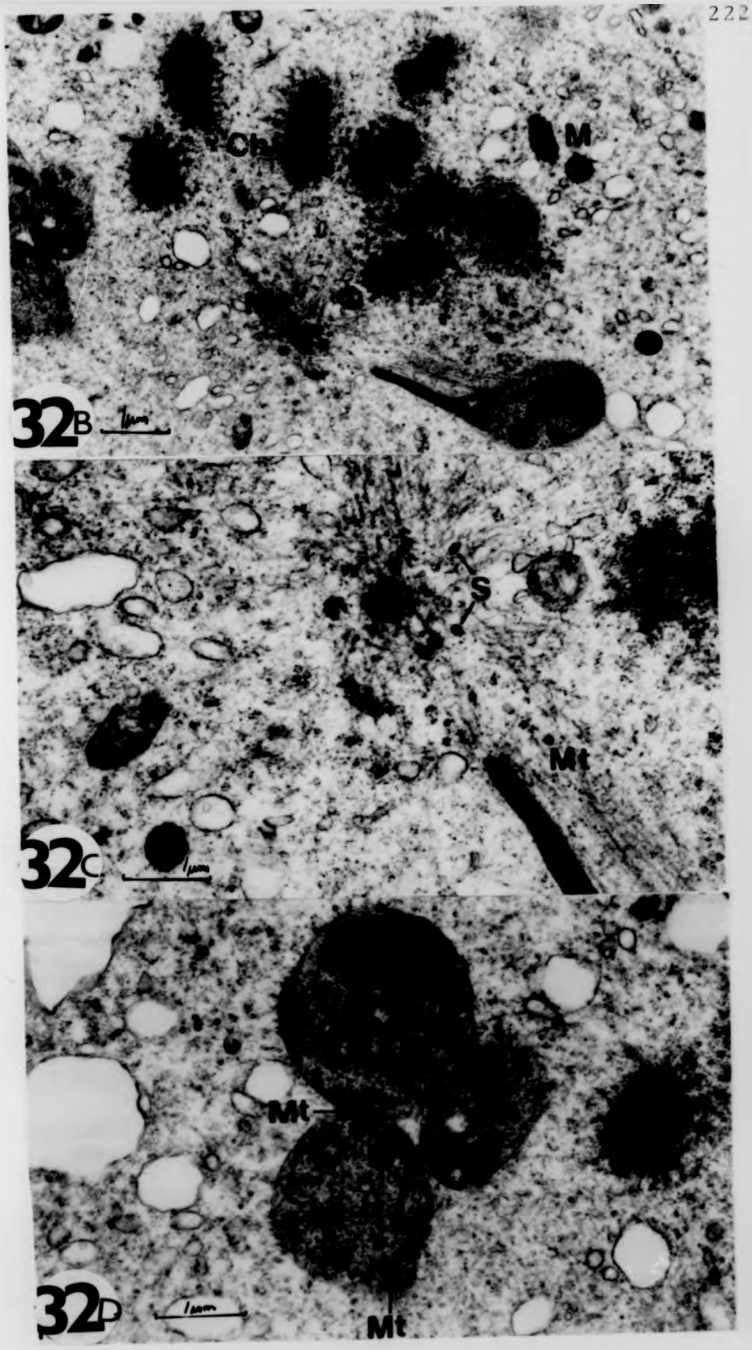


Fig. 32b: This is a higher magnification on Fig. 32a demonstrating the general arrangement of the Theileria and host cell chromosomes around the centriole.

Fig. 32c: This is a higher magnification (x36,000) of Fig. 32a showing details of centriole region and the microtubule arrangement.

Fig. 32d: A higher magnification (x15,000) showing microtubules running between and beyond parasite masses.



g. 32a
t of the
round

000) of
region

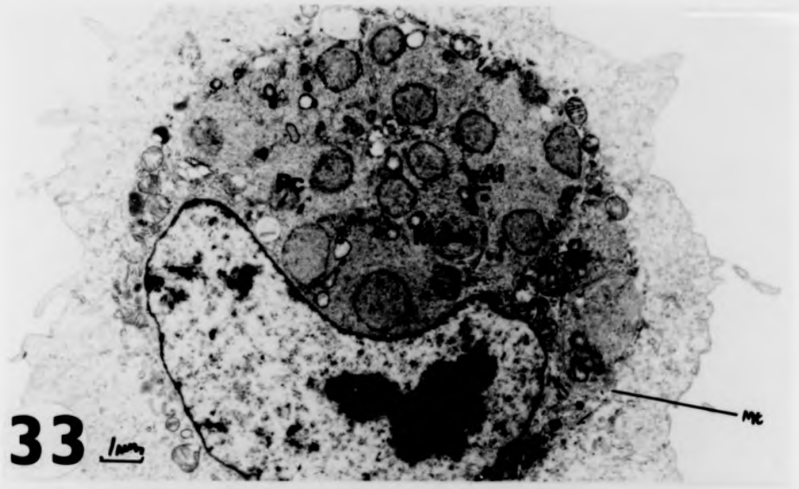
ving micro-
rasite

Fig. 33: A T. parva-infected lymphoblastoid cattle cell with several parasite masses. Note the massive number of microtubules extending from the parasites into lymphoblast cytoplasm and the single layer annulate lamella as well as mitotic spindle (Ms) in Theileria nucleus.

Fig. 34: A T. parva-infected lymphoblastoid cattle cell showing details of the intranuclear mitotic spindle of Theileria spp.

ttle cell
the massive
the
and the
as mitotic

ttle cell
nitotic



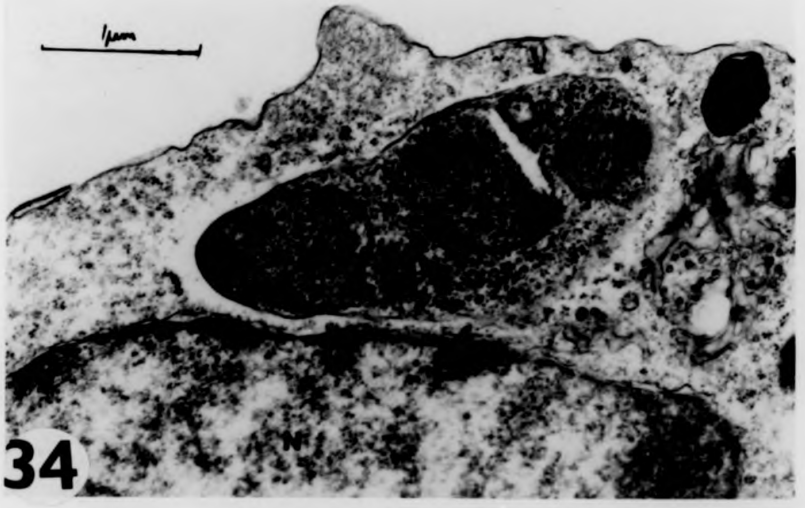
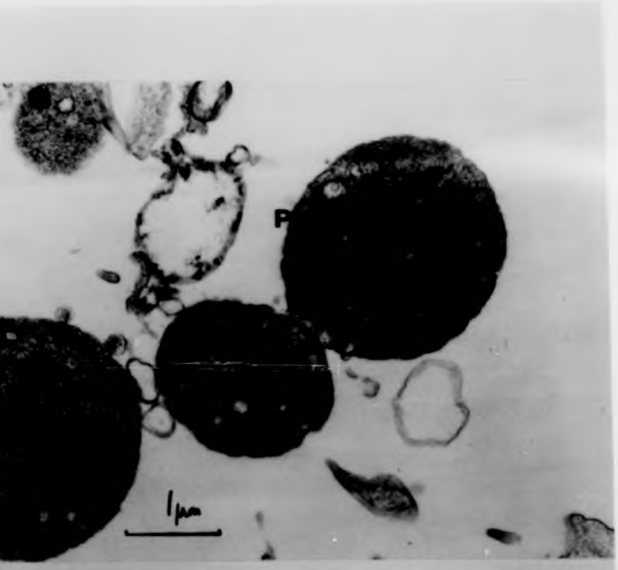


Fig. 35: Extra cellular schizonts of T. parva. Note the general morphology which is very similar to that of intracellular schizonts but they are more spherical than is often observed inside the lymphoblast.

Fig. 36a: A T. parva-infected lymphoblastoid cattle cell with intracellular Theileria merozoites

a. Note the
milar to that
are more
side the

cattle cell
ites


1μm

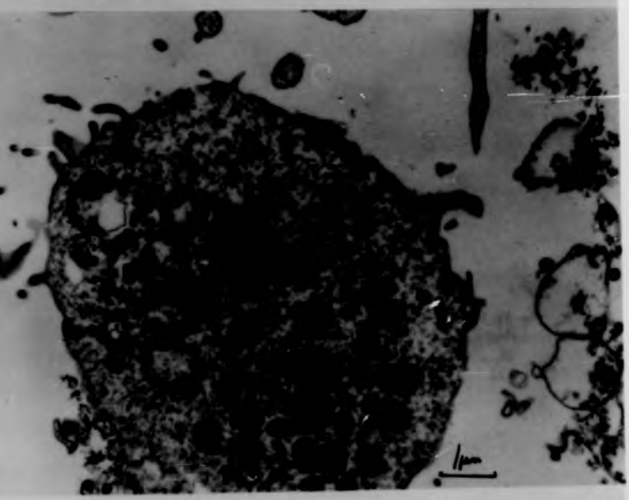

1μm

Fig. 36b: A higher magnification (x32,000) of Fig. 32a, showing the details of intralymphocytic Theileria merozoites.

Fig. 36c: A higher magnification (x80,000) of merozoites in Fig. 32a, demonstrating the fuzzy coat (Sc) surrounding merozoite limiting unit membrane.

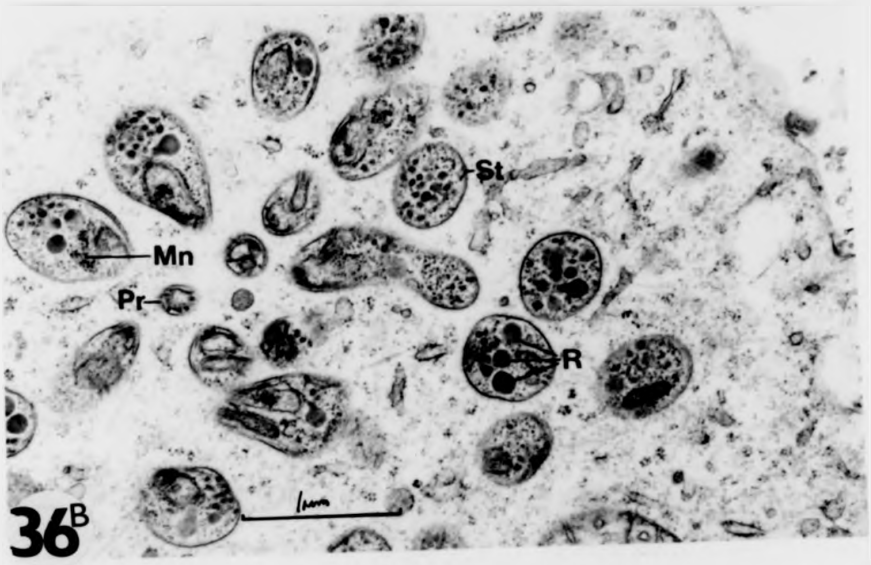
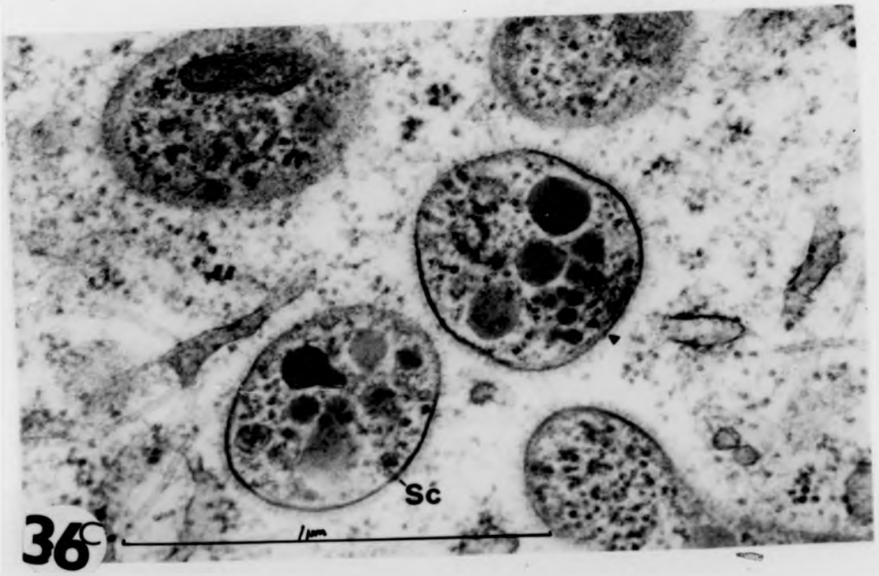


Fig. 32a,
ytic

merozoites
y coat (Sc)
memberane.



Key to Figs. 37-44

Tpc	*T. parva* in cattle cells
T1c	*T. lawrencei* in cattle cells
T1b	*T. lawrencei* in buffalo cells
Tac	*T. annulata* in cattle cells

1	cell line E 174
2	cell line L 81
3	cell line L 62
4	cell line L 89
5	cell line L 493
6	cell line 515
7	cell line W 325

ELECTROPHORETIC FORMS OF GLUCOSE PHOSPHATE ISOMERASE (GPI) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

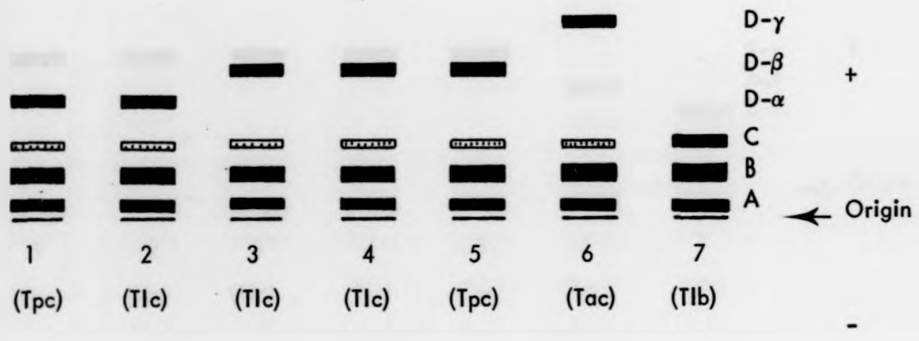


Fig. 37

ELECTROPHORETIC FORMS OF GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE (GAPDH) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

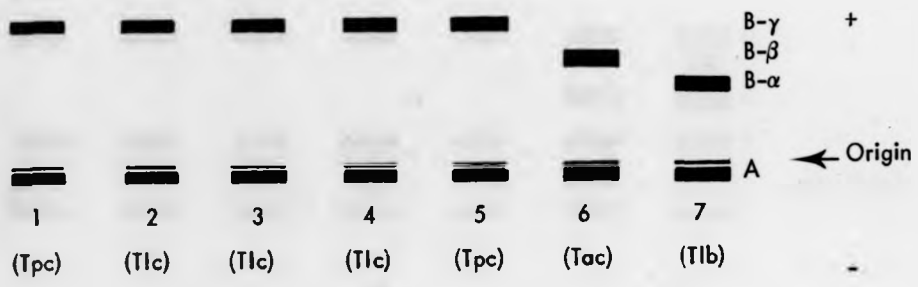


Fig. 38

ELECTROPHORETIC FORMS OF ALDOLASE (ALD) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

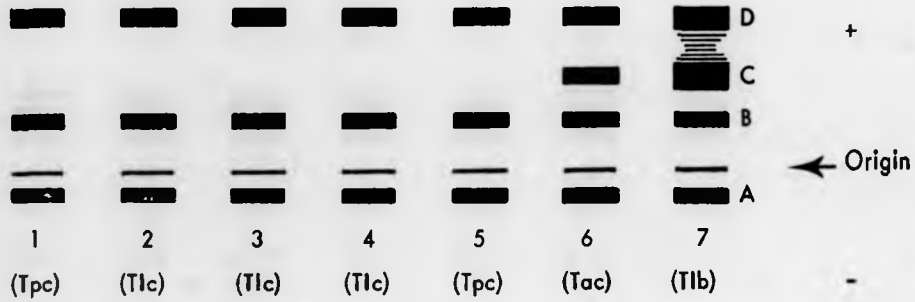


Fig. 39

ELECTROPHORETIC FORMS OF PHOSPHOGLUCOMUTASE (PGM) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

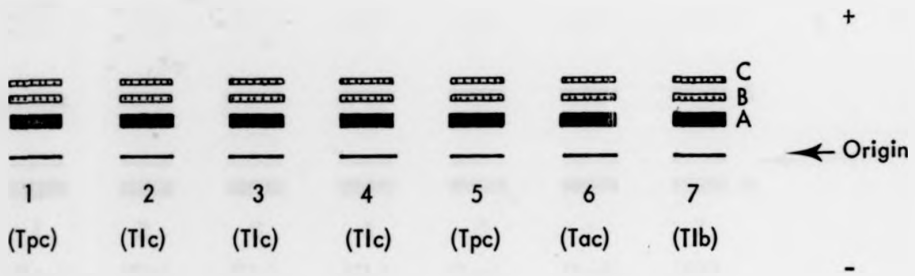


Fig. 40

ELECTROPHORETIC FORMS OF MALATE DEHYDROGENASE (MDH) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

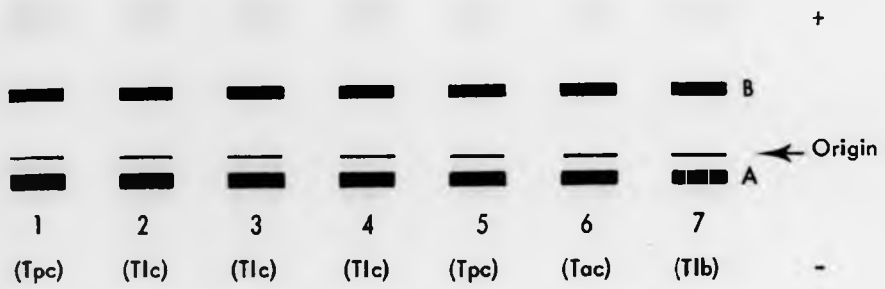


Fig. 41

ELECTROPHORETIC FORMS OF ASPARTATE AMINOTRANSFERASE (ASAT) IN THEILERIA -INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

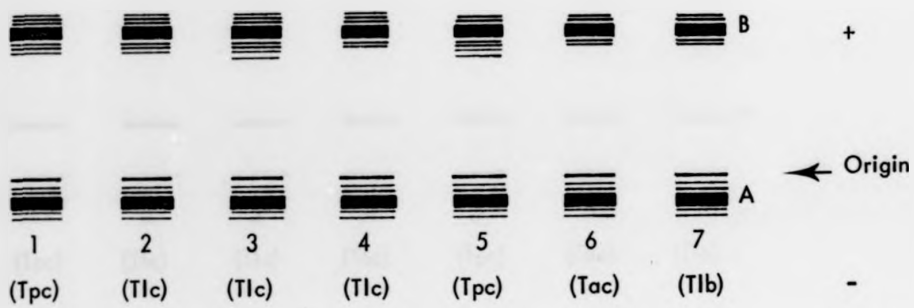


Fig. 42

ELECTROPHORETIC FORMS OF MALIC ENZYME (ME) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

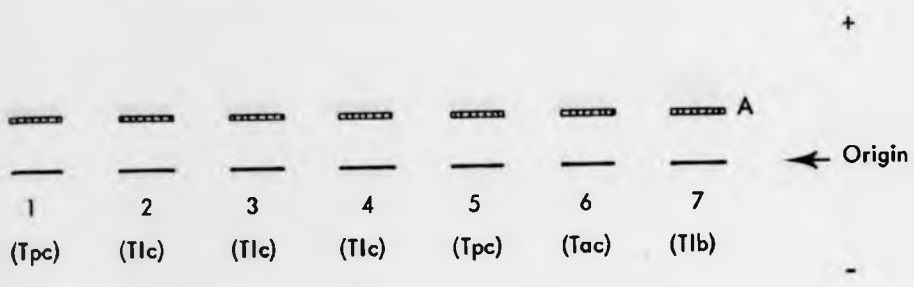


Fig. 43

ELECTROPHORETIC FORMS OF GLUCOSE 6 PHOSPHATE DEHYDROGENASE (G6PD) IN THEILERIA-INFECTED LYMPHOID CELLS OF BUFFALO AND CATTLE

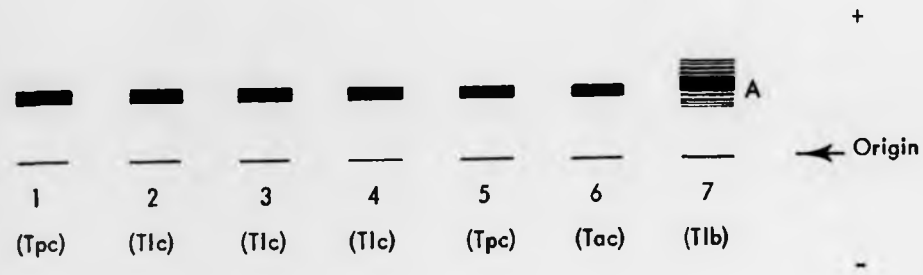


Fig. 44

Key to Figs. 45-47

CCC	Cattle control cells
BCC	Buffalo control cells
SCC	Cells from cell lines BL-3, 20, 24 or uninfected stimulated (Con A) lymphoid cells from primary cultures
T1c	*T. lawrencei*-infected cattle cells
T1b	*T. lawrencei*-infected buffalo cells
Tpc	*T. parva*-infected cattle cells
Tac	*T. annulata*-infected cattle cells
1	Lysates from unstimulated and LPS stimulated cells (Table XIX)
2	Lysate from uninfected buffalo cells (Table V)
3	Lysates from BL-3, 20, 24 and Con A stimulated lymphoid cells from primary cultures (Table XIX)
4	Lysates from cell line L 81
5	Lysates from cell line E 174
6	Lysates from cell line L 62
7	Lysates from cell line L 89
8	Lysates from cell line L 493
9	Lysates from cell line S 3
10	Lysates from cell line S 15
11	Lysates from cell line S 19/224
12	Lysates from cell line W 325

Key to Figs. 45-47

CCC	Cattle control cells
BCC	Buffalo control cells
SCC	Cells from cell lines BL-3, 20, 24 or uninfected stimulated (Con A) lymphoid cells from primary cultures
T1c	*T. lawrencei*-infected cattle cells
T1b	*T. lawrencei*-infected buffalo cells
Tpc	*T. parva*-infected cattle cells
Tac	*T. annulata*-infected cattle cells
1	Lysates from unstimulated and LPS stimulated cells (Table XIX)
2	Lysate from uninfected buffalo cells (Table V)
3	Lysates from BL-3, 20, 24 and Con A stimulated lymphoid cells from primary cultures (Table XIX)
4	Lysates from cell line L 81
5	Lysates from cell line E 174
6	Lysates from cell line L 62
7	Lysates from cell line L 89
8	Lysates from cell line L 493
9	Lysates from cell line S 3
10	Lysates from cell line S 15
11	Lysates from cell line S 19/224
12	Lysates from cell line W 325

A SUMMARY OF ELECTROPHORETIC PATTERNS OF GPI-ISOENZYMES FROM PARASITIZED AND NON-PARASITIZED LYMPHOID CELLS OF BUFFALO AND CATTLE.

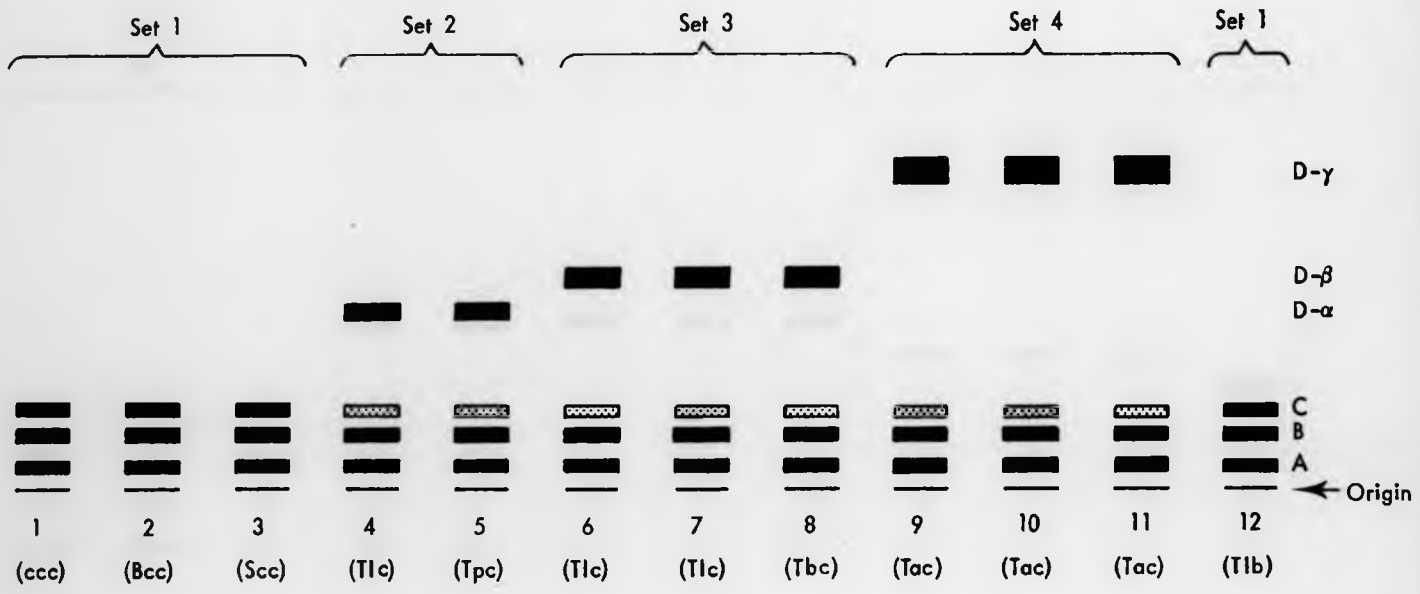


Fig. 45

A SUMMARY OF ELECTROPHORETIC PATTERNS OF GAPDH-ISOENZYMES FROM PARASITIZED AND NON-PARASITIZED LYMPHOID CELLS OF BUFFALO AND CATTLE.

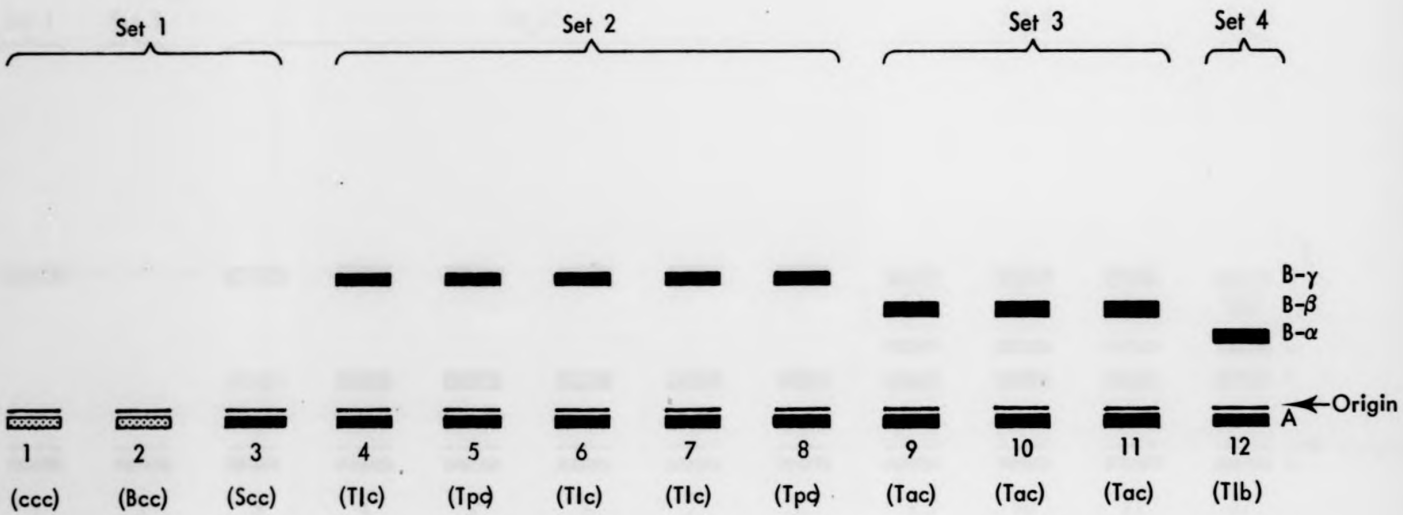


Fig. 46

A SUMMARY OF ELECTROPHORETIC PATTERNS OF ALD-ISOENZYMES FROM PARASITIZED AND NON-PARASITIZED LYMPHOID CELLS OF BUFFALO AND CATTLE.

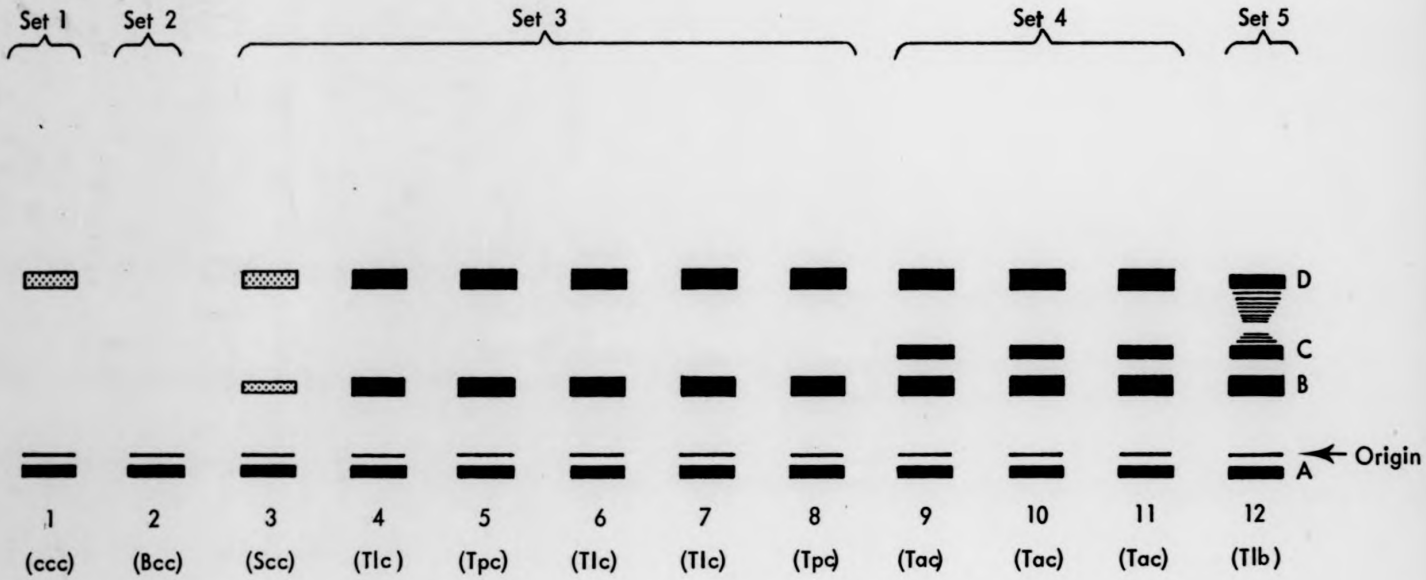


Fig.47

ACKNOWLEDGEMENTS

I am very grateful to Professor W.H.R. Lumsden and Dr C.G.D. Brown for their supervision and assistance in obtaining cell lines used in the research, Dr R.G. Bird for his helpful suggestions on electron microscopy and Dr V. Kilgour for her constructive criticism on the biochemical aspects.

I would also like to express my sincere thanks to all my colleagues on the former UNDP/FAO tickborne diseases project at Muguga, especially Drs M.P. Cunningham, A.S. Young and S.P. Morzaria. Their constant interest, enthusiasm and constructive criticisms provided ideal conditions for this work.

The diligent technical assistance provided by Mr Lawrence Njuguna at Muguga, Mr Michael Smith in the E.M. unit as well as Mr P. Sergeaunt and Mr J. Williams of the Department of Medical Protozoology in the School is very much appreciated. My thanks also go to Mr David Day for his invaluable help in preparation of the photographs.

The financial support by the British Council (TCTD) is very gratefully acknowledged.

Finally, I would like to record my appreciation of the encouragement and sacrifices made by my wife, son and parents.

REFERENCES

ANON. (1967)

Report of the East African Livestock Survey
UNDP FAO/SF 21 REG. Vols. 1 and 2, pp. 215 and 279.

ANON. (1976)

A review of recent progress in the control of ticks and tick-borne diseases in East Africa
FAO Report (AGA/MISC/76/1).

ANON. (1978)

Manual of tissue culture techniques
FAO Technical Report 3 (AG:DP/RAF/67/077)

ANON (1978)

Proposals for the nomenclature of salivarian trypanosomes and for the maintenance of reference collections
Bulletin of the World Health Organization 56(3): 467-480

ADLER, S. AND ELLENBOGEN, N. (1934)

A note on the pre-immunization of calves against Theileria annulata
Veterinary Record 14, 31-93.

ALLEN, S.L. (1960)

Inherited variations in the esterases of tetrahymena
Genetics 45, 1051-1070.

AL-TAQI, M. AND EVANS, D.A. (1978)

Characterization of Leishmania spp. from Kuwait by isoenzyme electrophoresis
Transactions of the Royal Society of Tropical Medicine and Hygiene 72, 56-65.

ASAKAROV, E.M. (1975)
Virulence and immunogenicity of Theileria annulata
Veterinariya (Moscow) No. 5, 76-78.

BAGSTER, I.A. AND PARR, C.W. (1973)
Trypanosome identification by electrophoresis
of soluble enzymes
Nature 244, 364-366.

BARNETT, S.F. (1957)
Theileriosis control
Bulletin of Epizootic Diseases of Africa 5,
343-357.

BARNETT, S.F. (1960)
Connective tissue reactions in acute fatal
East Coast Fever (Theileria parva) of cattle
Journal of Infectious Diseases 107, 253-282.

BARNETT, S.F. (1968)
Theileriasis
In "Infectious Blood Diseases of Man and Animals"
(Weinman, D. and Ristic, M., Eds.), Vol. 2,
pp. 269-328, Academic Press, New York.

BARNETT, S.F. (1977)
Theileria
In "Parasitic Protozoa" (Kreier, J.P., Ed.),
Vol. IV, pp. 77-114.

BARNETT, S.F. AND BROCKLESBY, D.W. (1966)
The susceptibility of the African buffalo
(Syncerus caffer) to infection with Theileria
parva (Theiler, 1904).
British Veterinary Journal 122, 379-386.

BETTENCOURT, A., FRANCA, C., BORGES, J. (1907)
Un cas de piroplasmose bacilleforme chez le daim. Archivos do R. Instituto Bacteriologico Camara Pestana, Lisboa 1, 341.

BHATTACHARYULU, Y., CHANDHRI, R., AND GILL, P. (1975)
Transstadial transmission of Theileria annulata through common ixodid ticks infesting Indian cattle
Parasitology 71, 1-7.

BROCKLESBY, D.W. (1962)
Cytauxzoon taurotragi Martin and Brocklesby, 1960, a piroplasm of the eland (Taurotragus oryx pattersonianus Lydekker, 1906)
Research in Veterinary Science 3, 334-344.

BROCKLESBY, D.W. (1966)
Theileridae of the buffalo in East Africa
Proceedings of the First International Congress of Parasitology (Corradetti, A. Ed.), I, 274-275.

BROCKLESBY, D.W. (1978)
Recent observations on tick-borne diseases
In "Tick-borne Diseases and their Vectors" (J.K.H. Wilde, Ed.), pp. 263-286.
Centre for tropical Veterinary Medicine, University of Edinburgh.

BROCKLESBY, D.W. AND BAILEY, K.P. (1965)
The immunization of cattle against East Coast Fever (Theileria parva infection) using tetracyclines: a review of the literature and a

reappraisal of the method
Bulletin of Epizootic Diseases of Africa 13, 161-168.

BROCKLESBY, D.W., BAILEY, K.P., JARRETT, W.F.H., MARTIN, W.B., MILLER, H.R.P., NDERITO, P. AND URQUHART, G.M. (1965) Experiments in immunity to East Coast Fever
Veterinary Record 77, 512.

BROCKLESBY, D.W., BARNETT, S.F. AND SCOTT, G.R. (1961) Morbidity and mortality rates in East Coast Fever (Theileria parva infection) and their application to drug screening procedures
British Veterinary Journal 117, 529-531.

BROCKLESBY, D.W. AND HAWKING, F. (1958) Growth of Theileria annulata and T. parva in tissue culture
Transactions of the Royal Society of Tropical Medicine and Hygiene 52, 414-420.

BROCKLESBY, D.W. AND VIDLER, B.O. (1961) Attempts to infect some small laboratory animals with Theileria parva
Research in Veterinary Science 3, 285-287.

BROCKLESBY, D.W., MORZARIA, S.P. and HARRADINE, D.L. (1975) Theileria mutans: Experimental Transmission by Haemaphysalis punctata
Tropenmedizin und Parasitologie 26, 295-302.

BROWN, C.G.D., CRAWFORD, J.G., KANHAI, G.K., NJUGUNA, L.M. AND STAGG, D.A. (1978) Immunization of cattle against East Coast Fever

with lymphoblastoid cell lines infected and transformed by Theileria parva
In "Tick-borne Diseases and Their Vectors" (J.K.H. Wilde, Ed.), pp. 331-333.

BROWN, C.G.D., MALMQUIST, W.A., CUNNINGHAM, M.P., RADLEY, D.E. AND BURRIDGE, M.J. (1970)
Immunization against East Coast Fever. Inoculation of cattle with Theileria parva schizonts grown in cell culture.
Journal of Parasitology 57 (4), 59-60.

BROWN, C.G.D., RADLEY, D.E., BURRIDGE, M.J. AND CUNNINGHAM, M.P. (1977)
The use of tetracyclines in the chemotherapy of experimental East Coast Fever (Theileria parva infection of cattle)
Tropenmedizin und Parasitologie 28, 513-520.

BROWN, C.G.D., STAGG, D.A., PURNELL, R.E., KANHAI, G.K. AND PAYNE, R.C. (1973)
Infection and transformation of bovine lymphoid cells in vitro by infective particles of Theileria parva
Nature, London, 245, 101-103.

BURRIDGE, M.J. (1975)
Role of wild mammals in the epidemiology of bovine theilerioses in East Africa
Journal of Wildlife Diseases 11, 68-72.

BURRIDGE, M.J., BROWN, C.G.D., CRAWFORD, J.G., KIRIMI, I.M., MORZARIA, S.P., PAYNE, R.C. AND NEWSON, R.M. (1974)
Preliminary studies on an atypical strain of bovine

Theileria isolated in Kenya
Research in Veterinary Science 17, 139-144.

BURRIDGE, M.J. AND KIMBER, C.D. (1972)
The indirect fluorescent antibody test for experimental East Coast Fever (*Theileria parva* infection in cattle). Evaluation of a cell culture schizont antigen
Research in Veterinary Science 13, 45-1455.

BURRIDGE, M.J. AND ODEKE (1973)
Theileria lawrencei infection in Indian water buffalo (*Bubalus bubalis*)
Experimental Parasitology 34, 257-261.

BÜTTNER, D.W. (1967a)
Die feinstruktur der merozoiten von *Theileria parva*
Tropenmedizin und Parasitologie 18, 224-244.

BÜTTNER, D.W. (1967b)
Elektronen mikrokopische studien der vermehrung von *Theileria parva* im rind
Tropenmedizin und Parasitologie 18, 245-268.

CARTER, R. (1970)
Enzyme variation in *Plasmodium berghei*
Transactions of the Royal Society of Tropical Medicine and Hygiene 64, 401-406.

CARTER, R. (1973)
Enzyme variation in *Plasmodium berghei* and *Plasmodium vinckei*
Parasitology 66, 297-307.

CHEMNITZ, J. AND SLAMBERG, K. (1978)
Observations on annulate lamellae and the cytoplasmic microtubule complex in tumor cells
Journal of Ultrastructure Research 63, 101-102.

COWDRY, E.V. AND DANKS, W.B.C. (1933)
Studies on East Coast Fever. II. Behaviour of the parasite and the development of distinctive lesions in susceptible animals
Parasitology 25, 1-63.

COWDRY, E.V. AND HAM, A.W. (1932)
Studies on East Coast Fever. I. Life Cycle of the parasite in ticks
Parasitology 24, 1-49.

CUNNINGHAM, M.P., BROWN, C.G.D., BURRIDGE, M.J., IRVIN, A.D., KIRIMI, I.M., PURNELL, R.E., RADLEY, D.E. AND WAGNER, G.G. (1974a)
Theileriosis: The exposure of immunized cattle in a Theileria lawrencei enzootic area
Tropical Animal Health and Production 6, 39-43.

CUNNINGHAM, M.P., BROWN, C.G.D., BURRIDGE, M.J., JOYNER, L.P. AND PURNELL, R.E. (1973a)
East Coast Fever: The infectivity for cattle of infective particles of Theileria parva harvested in various substrates
International Journal of Parasitology 3, 335-338.

CUNNINGHAM, M.P., BROWN, C.G.D., BURRIDGE, M.J., MUSOKE, A.J., PURNELL, R.F., RADLEY, D.E. AND SEMPEBWA, C. (1974c)
East Coast Fever: Titration in cattle of

suspensions of Theileria parva derived from ticks
British Veterinary Journal 130, 336-343.

CUNNINGHAM, M.P., BROWN, C.G.D., BURRIDGE, M.J. AND PURNELL, R.E. (1973b)
Cryopreservation of infective particles of Theileria parva
International Journal of Parasitology 3, 583-587.

DAWSON, R.M.C., ELLIOT, D.C., ELLIOT, W.H. AND JONES, K.M. (1969)
Definitions, formulae and general information
In "Data for Biochemical Research", p. 625.

DE KOCK, G. (1957)
Studies on the lesions and pathogenesis of East Coast Fever (Theileria parva infection) in cattle, with special reference to the lymphoid tissue
Onderstepoort Journal of Veterinary Research 27, 431-454.

DE LIMA, V.M.G., ROITMAN, I. AND KILGOUR, V. (1979)
Five trypanosomatid species of insects distinguished by isoenzymes
Journal of Protozoology (in press).

DE MARTINI, J.C. AND MOULTON, J.E. (1973a)
Responses of the bovine lymphatic system to infection by Theileria parva
I. History and ultrastructure of lymphnodes in experimentally infected calves
Journal of Comparative Pathology 83, 281-298.

DE MARTINI, J.C. AND MOULTON, J.E. (1973b)
Responses of the bovine lymphatic system to infection by Theileria parva
II. Changes in the central lymph in experimentally-

infected calves
Journal of Comparative Pathology 83, 299-306.

DSCHUNKOWSKY, E. AND LUHS, J. (1903, 1904)
Cited by Neitz, W.O. (1957) Theileriosis, Gonderioses and cytauxzoonoses
Onderstepoort Journal of Veterinary Research 27, 275-430.

DSCHUNKOWSKY, E. AND LUHS, J. (1909)
Protozoenkrankheiten des Blutes der Haustiere in Transkaukasien
9 Congr. Internat. Med. Vet. La Haye, p. 31.

DUFFUS, W.P.H. AND WAGNER, G.G. (1974)
Immunochemical studies on ECF: III. Development of an indirect haemaglutination assay using Theileria parva piroplasm antigen
Journal of Parasitology 60, 860-865.

DU TOIT (1918)
Zur systematik der piroplasmen
Archiv fUr Protistenkunde 39, 84-104.

DU TOIT (1930)
Theileriasis
Rept. Sect. Meetings 11th Internat. Vet. Congress, Vol. 3, 1-34.

D'YAKONOV, L.P., STEPANOVA, N.I., ASAKAROV, E. (1975)
Clinical and immunological status of cattle vaccinated against T. annulata
Trudy Vsesoyuznogo Institua Eksperimental'noi Veterinarnii 43, 234-240.

FRANCA, C. (1909)
Cited by Weitz, W.O. (1957)
Onderstepoort Journal of Veterinary Research 27, 275-430.

GARDENER, P.J., CHANCE, M.L. AND PETERS, W. (1974)
Biochemical taxonomy of Leishmania
II. Electrophoretic variation of malata dehydrogenase
Annals of Tropical Medicine and Parasitology 68, 317-325.

GIBSON, W., MEHLITZ, D., LANHAM, S.M. AND GODFREY, D.G. (1978)
The identification of Trypanosoma brucei gambiense in Liberian pigs and dogs by isoenzyme and resistance to human plasma
Tropenmedizin und Parasitologie 29, 335-345.

GILL, B.S., BHATTACHARYULU, Y., KAUR, D. (1976)
Immunization against bovine tropical theileriosis (Theileria annulata infection)
Research in Veterinary Science 21, 146-149.

GODFREY, D. AND KILGOUR, V.K. (1976)
Enzyme electrophoresis in characterizing the causative organism of Gambian trypanosomiasis
Transactions of the Royal Society of Tropical Medicine and Hygiene 70, 219-224.

GONDER, R. (1910)
The life cycle of Theileria parva, the cause of East Coast Fever in cattle in South Africa
Journal of Comparative Pathology and Therapeutics 23, 328-335.

GUILBRIDE, P.D.L. AND OPWATA, B. (1963)
Observations on resistance of Jersey/Nganda calves to East Coast Fever (Theileria parva)
Bulletin of Epizootic Diseases of Africa 11, 289-298.

HAMMOND, D.M. (1973)
Life cycles and development of coccidia
In "The Coccidia" (Hammond, D.M. with Long, P.L., Eds.), pp. 45-79 (Butterworths, London).

HARRIS, H. AND HOPKINSON, D.A. (1976)
Handbook of enzyme electrophoresis in human genetics
North-Holland Publishing Co., Amsterdam.

HASHEMI-FESHARKI, R. AND SHAD-DEL, F. (1973)
Vaccination of calves and milking cows with different strains of Theileria annulata
American Journal of Veterinary Research 34, 1465-1467.

HOOSHMAND-RAD, P. AND HASHEMI-FESHARKI, R. (1968)
The effect of virulence on cultivation of Theileria annulata strains in lymphoid cells which have been cultured in suspension
Archives Institute Razi 20, 85-89.

HULLIGER, L. (1965)
Cultivation of three species of Theileria in lymphoid cells in vitro
Journal of Protozoology 12, 649-655.

HULLIGER, L., BROWN, C.G.D. AND WILDE, J.K.H. (1966)
Transition of developmental stages of Theileria parva in vitro at high temperature
Nature 211, 328-329.

HULLIGER, L., WILDE, J.K.H., BROWN, C.G.D. AND TURNER, L. (1964)
Mode of multiplication of Theileria in cultures of bovine lymphocytic cells
Nature 203, 728-730.

IRVIN, A.D., BROWN, C.G.D., BURRIDGE, M.J., CUNNINGHAM, M.P., MUSOKE, A.J., PIERCE, M.A., PURNELL, R.E. AND RADLEY, D.E. (1972a)
A pathogenic theilerial syndrome of cattle in the Narok District of Kenya. I. Transmission studies
Tropical Animal Health and Production 4, 220-229.

IRVIN, A.D., BROWN, C.G.D. AND STAGG, D.A. (1975)
Theileria parva: Effects of irradiation on a culture of parasitized bovine lymphoid cells
Experimental Parasitology 38, 64-74.

IRVIN, A.D., BROWN, C.G.D., STAGG, D.A., KANHAI, G.K., KIMBER, C.D. AND RADLEY, D.E. (1976)
Adaptation and possible attenuation of Theileria parva-infected cells grown in irradiated mice
Journal of Comparative Pathology 86, 51-57.

IRVIN, A.D., PIERCE, M.A., PURNELL, R.E. AND KING, J.M. (1972b)
The possible role of the eland (Taurotragus oryx) in the epidemiology of East Coast Fever and other bovine theilerioses
The Veterinary Record 91, 513-517.

IRVIN, A.D. AND STAGG, D.A. (1977)
Theileria parva: Purine and pyrimidine metabolism and the action of folate antagonists in parasitized bovine lymphoid cells
Experimental Parasitology 41, 172-185.

ISHIHARA, T. (1968)
Bovine piroplasmosis in Japan
Japanese Agricultural Research, Quarterly, 3, 23-31.

JARRETT, W.F.H. AND BROCKLESBY, D.W. (1966)
A preliminary electron microscopic study of East Coast Fever (Theileria parva infection)
Journal of Protozoology 13, 301-310.

JARRETT, W.F.H., CRIGHTON, G.W. AND PIRIE, H.M. (1969)
Theileria parva: Kinetics of replication
Experimental Parasitology 24, 9-25.

KALTENBACH, J.P., KALTENBACH, M.H. AND LYONS, W.B. (1958)
Nigrosin as a dye for differentiating live and dead ascites cells
Experimental Cell Research 15, 112-117.

KELLENBERGER, E., RYTER, A. AND SECHAUD, J. (1958)
Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states
J. Biophysical and Biochemical Cytology 4, 671.

KILGOUR, V., GARDENER, P.J., GODFREY, D.G. AND PETERS, W. (1974)
Demonstration of electrophoretic variation of two aminotransferases in Leishmania
Annals of Tropical Medicine and Parasitology 68, 245-264.

KILGOUR, V. AND GODFREY, D.G. (1973)
Species-characteristic isoenzymes of two amino-transferases in trypanosomes
Nature New Biology 244, 69-70.

KILGOUR, V., GODFREY, D.G. AND NA'ISA, B.K. (1975)
Isoenzymes of two aminotransferases among Trypanosoma vivax in Nigerian cattle
Annals of Tropical Medicine and Parasitology 69, 329-335.

KIMBER, C.D. (1974)
The serology of theilerial parasites isolated in Kenya
Paper presented at the VII Specialist Committee Meeting on Veterinary Diseases Common to East African countries. Held by the E.A. Community at EAVRO, Muguga, 18-20 September, 1974.

KIMBER, C.D. AND BURRIDGE, M.J. (1972)
The indirect fluorescent antibody test for experimental East Coast Fever (Theileria parva infection of cattle). Evaluation of blood samples as a source of antibody
Research in Veterinary Science 13, 133-135.

KIMETO, B.A. (1976)
Ultrastructure of blood platelets in cattle with East Coast Fever
American Journal of Veterinary Research 37, 443-447.

KLUGE, E., CANHAM, A.S. AND NEITZ, W.O. (1956)
Cited by Neitz, W.O. (1957)
Ondesterpoort Journal of Veterinary Research 27, 275-430.

KOCH, R. (1897)
Cited by Neitz, W.O., 1957
In "Theileriosis, Gonderiosis and Cytauxzoonoses"
Onderstepoort Journal of Veterinary Research 27, 275-430.

KOCH, R. (1903)
Rhodesian redwater or African Coast Fever
Journal of Comparative Pathology and Therapeutics 16, 390-398.

KOCH, R. (1906)
Beitrage zur entwickelung der piroplasmen
Zeitschrift für Hygiene 54, 1-7.

LAWRENCE, D.A. (1933)
African Coast Fever and Theileriosis
Southern Rhodesia, Report by Director of Veterinary Research for the year 1933.

LAWRENCE, D.A. (1934)
Theileriosis
Southern Rhodesia, Report of the Director of Veterinary Research for the year 1934.

LEVINE, N.D. (1970)
Taxonomy of the sporozoa
Journal of Parasitology 56 (II), 208-209.

LEVINE, N.D. (1971)
Taxonomy of the piroplasms
Transactions of American Microscopy Society 90, 2-23.

LEWIS, E.A. (1943)
Cited by Neitz, W.O. (1957)
Ondesterpoort Journal of Veterinary Research 27, 275-430.

LÖHR, K.F. AND ROSS, J.P.J. (1969)
Serological response in cattle to East Coast Fever (Theileria parva infection) as measured by the indirect fluorescent antibody test
Research in Veterinary Science 10, 453.

LUMSDEN, W.H.R. AND HARDY, G.J.C. (1965)
Nomenclature of living parasite material
Nature, London 205, 1032.

LUMSDEN, W.H.R., ROBERTSON, D.H.H. AND McNEILLAGE, G.J.C. (1966)
Isolation, cultivation, low temperature preservation and infectivity titration of Trichomonas vaginalis
British Journal of Venereal Diseases 42, 145-154.

MACPHERSON, I. AND STOKER, M. (1962)
Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence
Virology 16, 147-151.

McHARDY, N., HAIG, A.J.B. AND DOLAN, T.T. (1976)
Chemotherapy of Theileria parva infection
Nature 261, 698-699.

MALING, H.M., WEBSTER, M.E., WILLIAMS, M.A., SAUL, W. AND ANDERSON, W. (1974)
Inflammation introduced by histamine, sertomin, bradykinin and compound 48/80. Antagonists and mechanism of action
Journal of Pharmacology and Experimental Therapeutics 191, 300-310.

MALMQUIST, W.A. AND BROWN, C.G.D. (1974)
Establishment of Theileria parva infected lymphoblastoid cell lines using homologous feeder layers
Research in Veterinary Science 16, 134-135.

MALMQUIST, W.A., NYINDO, M.B.A. AND BROWN, C.G.D. (1970)
East Coast Fever. Cultivation in vitro of bovine spleen cell lines infected and transformed by Theileria parva
Tropical Animal Health and Production 2, 139-145.

MALMQUIST, W.A., VAN DER MAATEN, M.J. AND BOOTH, A.D. (1969)
Isolation, immunodiffusion, immunofluorescence and electron microscopy of a syncytial virus lymphosarcomatous and apparently normal cattle
Cancer Research 29, 188-200.

MARTIN, H.M., BARNETT, S.F. AND VIDLER, B.O. (1964)
Cyclic development and longevity of Theileria parva in the tick Rhipicephalus appendiculatus
Experimental Parasitology 15, 527-555.

MAUL, G. (1968)
On the origin of annulate lamellae in human melanoma cells in vitro
Journal of Cell Biology 39, 88a (Abstract).

MERKOW, L. SLIFKIN, M. PARDO, M. AND ROMANO, O (1968)
Journal of Cell Biology 39, 89a-90a.

MEHLHORN, H., SCHEIN, E. AND WARNECKE, M. (1978)
Electron microscopic studies on the development of kinetes of Theileria parva. Theiler 1904 in the gut of the vector ticks Rhipicephalus appendiculatus Neumann, 1901
Acta Tropica 35, 123-136.

MILES, M.A., TOYE, P.J., OSWALD, S.C. AND GODFREY, D.G. (1977)
The identification by isoenzyme patterns of two distinct strain groups of Trypanosoma cruzi circulating independently in a rural area of Brazil
Transactions of the Royal Society of Tropical Medicine and Hygiene 71, 217-225.

MIRZABEKOV, D.A., AGAEV, A.A., MOVSUMZADE, A.K. AND GODZHAEV, A.N. (1969)
Immunization of cattle against theileriosis (T. annulata)
Veterinariya (Moscow) No. 3, 46-43.

MORZARIA, S.P., YOUNG, A.S. AND KIMBER, C.D. (1977)
The serological relationship of a British Theileria with other Theileria species using the indirect fluorescent antibody test
Research in Veterinary Science 22, 330-333.

MOULTON, J.E., KRAUSS, H.H. AND MALMQUIST, W.A. (1971a)
Transformation of reticulum cells to lymphoblasts in cultures of bovine spleen infected with Theileria parva
Laboratory Investigation 24, 187-196.

MOULTON, J.E., KRAUSS, H.H. AND MALMQUIST, W.A. (1971b)
Growth characteristics of Theileria parva-infected bovine lymphoblast cultures
American Journal of Veterinary Research 32, 1365-1370.

MUGERA, G.M. AND MUNYUA, W.K. (1973)
A study of developmental stages of Theileria parva by electron microscopy
Bulletin of Epizootic Diseases of Africa 21, 51-56.

NEITZ, W.O. (1940)
Cited by Neitz, W.O. (1957)
Onderstepoort Journal of Veterinary Research 27, 275-430.

NEITZ, W.O. (1953)
Aureomycin in Theileria parva infection
Nature 171, 34-35.

NEITZ, W.O. (1955)
Corridor disease: A fatal form of bovine theileriosis encountered in Zululand
Bulletin of Epizootic Diseases of Africa 3, 121-123.

NEITZ, W.O. (1957)
Theileriosis, gonderioses and cytauxzooneses
Onderstepoort Journal of Veterinary Research 27, 275-430.

NEITZ, W.O. (1963)
Review of recent developments in the protozoology of tickborne diseases
Second Meeting FAO/OJE Panel, Tick-borne Diseases Cario, 1962, pp. 34-35.

NEITZ, W.O. (1964)
The immunity in East Coast Fever
Journal of South African Veterinary Medical Association 35, 5-6.

NEITZ, W.O., ADELAAR, T.F. AND KLUGE, E. (1953)
Cited by Neitz, W.O. (1957)
Onderstepoort Journal of Veterinary Research 27, 275-430.

NEITZ, W.O. CANHAM, A.S. AND KLUGE, E. (1955)
Corridor disease: A fatal form of bovine theileriosis encountered in Zululand
Journal of South African Veterinary Medical Association 26, 79-87.

NEITZ, W.O. AND JANSEN, B.C. (1956)
A discussion on the classification of the Theileridae
Onderstepoort Journal of Veterinary Research 27, 7-18.

OBOLDOUEFF, G. AND GLAOUZO, J. (1928)
La theileriose bovides en Asie Centrale
Annales de l'Institut Pasteur 42, 1470-1479.

PAPPAS, G.D. (1959)
Electron microscope studies on amoebae
Annals of the New York Academy of Sciences 78, 448-473.

PETERS, J.H. (1975)
Preparation of large quantities of pure bovine lymphocytes and a monolayer for lymphocyte cultivation

In "Methods in Cell Biology" (Prescott, D.M., Ed.), 9, 1-11. Academic Press, New York.

PIPANO, E. (1976)
Basic principles of Theileria annulata control In "Theileriosis: Report of a workshop held in Nairobi, Kenya, 7-9 December 1976. Ottawa, International Development Research Centre, IDRC - 086e" (Henson, J.B. and Campbell, M., Eds.) pp. 55-65.

PIPANO, E., GOLDMAN, M., SAMISH, M. AND FRIEDHOFF :K.T. (1977)
Immunization of cattle against Theileria annulata using killed schizont vaccine
Veterinary Parasitology 3, 11-22.

PIPANO, E. AND TSUR, I. (1966)
Experimental immunization against Theileria annulata with tissue culture vaccine. I. Laboratory trials
Refuah Veterinarith 23, 133-136.

PIRIE, H.M., JARRETT, W.F.H. AND CRIGHTON, G.W. (1970)
Studies on vaccination against East Coast Fever using macroschizonts
Experimental Parasitology 27, 343-349.

POLLIACK, A., LAMPEN, N. AND DE HARVEN, E. (1974)
Scanning electron microscopy of lymphocytes of known B and T derivation
In "Proceedings of Workshop on advances in biomedical application of the scanning electromicroscope", I.I.T. Research Institute, Chicago, Illinois, USA.

In "Methods in Cell Biology" (Prescott, D.M., Ed.), 9, 1-11. Academic Press, New York.

PIPANO, E. (1976)
Basic principles of Theileria annulata control In "Theileriosis: Report of a workshop held in Nairobi, Kenya, 7-9 December 1976. Ottawa, International Development Research Centre, IDRC - 086e" (Henson, J.B. and Campbell, M., Eds.) pp. 55-65.

PIPANO, E., GOLDMAN, M., SAMISH, M. AND FRIEDHOFF : K.T. (1977)
Immunization of cattle against Theileria annulata using killed schizont vaccine
Veterinary Parasitology 3, 11-22.

PIPANO, E. AND TSUR, I. (1966)
Experimental immunization against Theileria annulata with tissue culture vaccine. I. Laboratory trials
Refuah Veterinarith 23, 133-136.

PIRIE, H.M., JARRETT, W.F.H. AND CRIGHTON, G.W. (1970)
Studies on vaccination against East Coast Fever using macroschizonts
Experimental Parasitology 27, 343-349.

POLLIACK, A., LAMPEN, N. AND DE HARVEN, E. (1974)
Scanning electron microscopy of lymphocytes of known B and T derivation
In "Proceedings of Workshop on advances in biomedical application of the scanning electromicroscope", I.I.T. Research Institute, Chicago, Illinois, USA.

PURNELL, R.E. (1977)
East Coast Fever: Some recent research in East Africa
Advances in Parasitology 15, 83-125.

PURNELL, R.E., BRANAGAN, D. AND BROWN, C.G.D. (1970)
Attempted transmission of some piroplasms by rhipicephalid ticks
Tropical Animal Health and Production 2, 146-150.

PURNELL, R.E. AND JOYNER, L.P. (1968)
The development of Theileria parva in the salivary glands of the tick Rhipicephalus appendiculatus
Parasitology 58, 725-732.

PURNELL, R.E., YOUNG, A.S., PAYNE, R.C. AND MWANGI, J.M. (1975)
Development of Theileria mutans (Aitomg) in the tick Amblyomma variegatum compared to that of T. parva (Muguga) in Rhipicephalus appendiculatus
Journal of Parasitology 61, 725-729.

RADLEY, D.E., BROWN, C.G.D., BURRIDGE, M.J., CUNNINGHAM, M.P., PEIRCE, M.A. AND PURNELL, R.E. (1974)
East Coast Fever: Quantitative studies of Theileria parva in cattle
Experimental Parasitology 36, 278-287.

RADLEY, D.E., BROWN, C.G.D., CUNNINGHAM, M.P., KIMBER, C.D., MUSISI, F.L., PAYNE, R.C., PURNELL, R.E., STAGG, S.M. AND YOUNG, A.S. (1975a)
East Coast Fever: 3. Chemoprophylactic immunization

of cattle using a combination of theilerial strains
Vaterinary Parasitology 1, 51-60.

RADLEY, D.E., YOUNG, A.S., BROWN, C.G.D., BURRIDGE, M.J., CUNNINGHAM, M.P., MUSISI, F.L. AND PURNELL, R.E. (1975a)
East Coast Fever: 2. Cross-immunity trials with a Kenya strain of Theileria lawrencei
Veterinary Parasitology 1, 43-50.

REEVES, R.E. AND BISCHOFF, J.M. (1968)
Classification of Entamoeba species by means of electrophoretic properties of amebal enzymes
Journal of Parasitology 54, 594-600.

REICHENOW, E. (1940)
Der entwickelungsgang des küstenfieber-erregers im rinde und in der übertragenden zecke
Archiv für Protistenkunde 94, 1-56.

ROUSE, B.T. AND BABUIK, L.A. (1974)
Host responses to infectious bovine rhinotracheitis virus. III. Isolation and immunologic activities of bovine T lymphocytes
Journal of Immunology 113, 1391-1397.

SAMISH, M. (1977)
Infective Theileria annulata in the host tick without a blood meal stimulus
Nature, U.K. 270, 51-52.

SCHEIN, E. (1975)
On the life cycle of Theileria annulata (Dschunskowsky and Luhs, 1904) in the midgut and

haemolymph of Hyalomma anatolicum excavatum (Koch, 1844)
Zeitschrift fÜr Parasitekunde 47, 165-167.

SCHEIN, E., MEHLHORN, H. AND WARNECKE, M. (1978)
Electron microscopic studies on the Schizogony of four Theileria species of cattle (T. parva, T. lawrencei, T. annulata and T. mutans)
Parasitologica 14, 337-348.

SCHEIN, E., WARNECKE, M. AND KIRMSE, P. (1977)
Development of Theileria parva (Theiler, 1904) in the gut of Rhipicephalus appendiculatus (Neumann, 1901)
Parasitology 75, 309-316.

SCHINDLER, R., MEHLITZ, D. AND MATSON, B. (1969)
Serological and immunological studies on Theileria lawrencei infection in cattle
Tropenmedizin und Parasitologie 20, 162-185.

SCHINDLER, R. AND WOKATSCH, R. (1965)
Attempts at differentiation of Theileria species from cattle by serological tests
Tropenmedizin und Parasitologie 16, 17-23.

SCHOLTYSECK, E. (1973)
Ultrastructure
In "The Coccidia" (Hammond, D.M. and Long, P.L., (Eds.), pp. 81-144, Butterworths, London.

SERGENT, E., DONATIEN, A., PARROT, L., LESTOQUARD, F., PLANTUREUX, E. AND ROUGEBIEF, H. (1924)
Les piroplasmoses bovines d'Algerie. Premier memoire
Archives de l'Institut Pasteur d'Algerie 2, 1-146.

SERGENT, E., DONATIEN, A., PARROT, L. AND LESTOQUARD, F. (1932)
Suppression experimentelle de la reproduction sennee chez un hamatozoaire Theileria dispar
Compte rendu hebdomadaire des seances de l'Academie des sciences, Paris, 195, 1054-1056.

SERGENT, E., DONATIEN, A., PARROT, L. AND LESTOQUARD, F. (1936)
Etude morphologique du cycle evolutief de Theileria dispar chez le boeuf et chez la tique
Annales de l'Institut Pasteur, Paris, 57, 30-55.

SMITH, K., IRVIN, A.D. AND STAGG, A.D. (1976)
An ultrastructural study of heterokaryons derived from Theileria parva infected bovine lymphoblasts and Erhlich ascites tumor cells
Research in Veterinary Science 21, 205-214.

SMITHIES, O. (1955)
Zone electrophoresis in starch gels; group variations in the serum proteins of normal human adults
Biochemistry Journal 61, 624-641.

SMITHIES, O. AND HICKMAN, C.G. (1958)
Inherited variations in the serum proteins of cattle
Genetics 43, 374-385.

SMITHIES, O. AND WALKER, N.F. (1955)
Genetic control of some serum proteins in normal humans
Nature 176, 1265-1266.

STAGG, D.A., BROWN, C.G.D., CRAWFORD, J.G., KANHAI, G.K. AND YOUNG, A.S. (1974)
In vitro cultivation of Theileria lawrencei-infected lymphoblastoid cell lines derived from a buffalo (Syncerus caffer)
Research in Veterinary Science 16, 125-127.

STEPANOVA, N.I., ZABLOTSKII, V.T., MUTUZKINA, Z.P., RASULOV, I.K., UMAROV, I.S. AND TUKHTAEV, B.T. (1977)
Live cell culture vaccine against Theileria annulata infection
Veterinariya (Moscow) USSR, No. 3, pp. 69-70.

STEPHENS, J.W.W. AND CHRISTOPHER, S.R. (1903)
The practical study of malaria and other blood parasites
New York, Longmans, Green, pp. 331-332.

THEILEN, G.H., RUSH, J.D., NELSON-REES, W.A., DUNGWORTH, D.L., MUNN, R.J. AND SWITZER, J.W. (1968)
Bovine leukemia: Establishment and morphologic characterization of contonuous cell suspension culture BL-1
Journal of the National Cancer Institute 40, 737-749.

THEILER, A. (1903)
Rhodesian tick fever
Transvaal Agricultural Journal 1, 93-110.

THEILER, A. (1904)
East Coast Fever
Transvaal Agricultural Journal 2, 421-438.

THEILER, A. (1906)
Piroplasma mutans (n. sp.) of South African cattle
Journal of Comparative Pathology and Therapeutics 19, 292-300.

THEILER, A. (1907)
Further notes on Piroplasma mutans, a new species of piroplasm in South African cattle
Journal of Comparative Pathology and Therapeutics 20, 1-18.

THEILER, A. AND GRAF, H. (1928)
Gonderia mutans or Theileria mutans?
13th and 14th Reports by the Director of Veterinary Education and Research, Union of South Africa, pp. 79-106.

TSUR, I. (1945)
Multiplication in vitro of Koch bodies of Theiliera annulata
Nature 156, 391.

TSUR, I. (1953)
Theileria annulata et Leishmania en culture de tissu
Proceedings of 15th International Veterinary Congress, Stockholm, Part I, pp. 26-31.

TSUR, I. AND ADLER, S. (1963)
Growth of Theileria annulata schizonts in a monolayer tissue culture
Journal of Protozoology (Supplement) 10 (3), 36.

TSUR, I., NEITZ, W.O. AND POLS, J.W. (1957)
The development of Koch bodies of Theileria parva in tissue culture
Refuah Veterinarith 14, 53-6.

TUTUSHIN, M.I. (1966)
Haemaglutination and haemaglutination inhibition tests for Theileria annulata infection
Veterinariya (Moscow) 43 (6), 46-48.

UILENBERG, G., McGREGOR, W., MPANGALA, C., CALLOW, L.L., DE VOS, A.J. (1978)
Relationships of some Theileria species of cattle
In "Tick-borne diseases and their vectors" (Wilde, J.K.H., Ed.), pp. 302-306.

VENABLE, J.H. AND COGGESHALL, R. (1965)
A simplified lead citrate stain for use in electron microscopy
Journal of Cell Biology 25, 407-408.

WENYON, C.M. (1926)
Protozoology
Bailliere, Tindall and Cox, London.

WILDE J.K.H. (1967)
East Coast Fever
Advances in Veterinary Science 11, 207-259.

WILDE, J.K.H., BROWN, C.G.D., HULLIGER, L., Gall, D. AND MACLEOD, W.G. (1968)
East Coast Fever: Experiments with the tissues of infected ticks
British Veterinary Journal 124, 196-208.

WISCHNITZER, S. (1970)
The annulate lamellae
International Review of Cytology 27, 65-100.

YAKIMOFF, W.L. AND DEKHTEREFF, N.A. (1930)
Zur frage über die Theileriose in Ostsibirien
Archiv für Prostistenkunde 72, 176-189.

YOUNG, A.S., GROOTENHUIS, J.G., BROWN, C.G.D., CUNNINGHAM, M.P., BURRIDGE, M.J., PURNELL, R.E. AND STAGG, D.A. (1978 a)
The incidence of Theileria species in East African buffalo (Syncerus caffer)
Tropenmedizin und Parasitologie 29, 281-288.

YOUNG, A.S., BROWN, C.G.D., BURRIDGE, M.J., CUNNINGHAM, M.P., PAYNE, R.C. AND PURNELL, R.E. (1977 a)
The establishment of an experimental field population of Theileria lawrencei-infected ticks maintained by African buffalo (Syncerus caffer)
Journal of Parasitology 63, 903-907.

YOUNG, A.S., BROWN, C.G.D., CUNNINGHAM, M.P. AND RADLEY, D.F. (1978b)
Evaluation of immunization of cattle against Theileria lawrencei
In "Tick-borne Diseases and their Vectors" (Wilde, J.K.H., Ed.), pp. 293-296.

YOUNG, A.S. AND PURNELL, R.E. (1973)
Transmission of Theileria lawrencei (Serengeti) by the ixodid tick Rhipicephalus appendiculatus
Tropical Animal Health Production 5, 146-152.

YOUNG, A.S., GROTENHUIS, J.G., KIMBER, C.D., KANHAI, G.K. AND STAGG, D.A. (1977b)
Tropenmedizin und Parasitologie 28, 185-194.

YOUNG, A.S., PURNELL, R.E., KIMBER, C.D. AND PAYNE, R.C. (1975)
Correlation between the morphology and infectivity of Theileria lawrencei developing in the tick Rhipicephalus appendiculatus
Parasitology 71, 27-34.

YOUNG, A.S., PURNELL, R.E., PAYNE, R.C., BROWN, C.G.D. AND KANHAI, G.K. (1978)
Studies on the transmission of a Kenyan strain of Theileria mutans
Parasitology 76, 99-115.

ZABLOTSKII, V.T. (1967)
Use of tissue culture in the study of Theileria annulata
Veterinariya (Moscow) 9, 66-69.

ADDENDA

YOUNG, A.S., BROWN, C.G.D., BURRIDGE, M.J., CUNNINGHAM, M.P., KIRIMI, I., AND IRVIN, A.D. (1973)
Observations on the crossimmunity between Theileria lawrencei (Serengeti) and Theileria parva (Muguga) in cattle.
International Journal for Parasitology 3, 723-728.

YOUNG, A.S., GROOTENHUIS, J.G., KIMBER, G.K., AND STAGG, D.A. (1977)
Isolation of Theileria species from eland (Taurotragus oryx) infective for cattle.

YOUNG, A.S., BURRIDGE, M.J. AND PAYNE, R. C. (1977)
Transmission of a Theileria species to cattle by the ixodid tick Amblyomma cohaerens, Donitz 1909
Tropical Animal Health and Production, 9, 37-45.