# STUDIES ON SUSPENSION CULTURES OF LYMPHOID CELLS INFECTED WITH *THEILERIA ANNULATA* (DSCHUNKOWSKY AND LUHS 1904)

by

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## SUMMARY

*Theileria annulata* infection is a tick-borne protozoal disease of cattle. The high mortality and lack of any effective treatment make this disease one of great economic importance.

The success achieved in adaptation of the parasite to culture *in vitro* facilitated investigation of the disease and the consequent attenuation of the parasite led to the production of experimental vaccines.

The literature relevant to the aspects studied was reviewed. Methods of isolation and adaptation of strains of the parasite in tissue culture were compared. A simple and new method of isolation from whole blood was introduced. The first isolation of a very virulent strain in tissue culture was achieved.

Growth rates of five strains of *T. annulata in vitro* showed an inverse relationship between rate of growth and virulence of strain. Several growth media and supplements were compared and the most satisfactory were shown to be Eagle's MEM with calf serum, lactalbumin hydrolysate and yeast extract. Growth of *T. annulata*-infected cells in various conditions was measured and factors affecting it were investigated. Of these, pH of the medium and percentage of viable cells in suspension were very important.

For morphological studies, from several methods of preparation of smears and staining, a modified method of linear smears was adopted. The morphology of the macroschizont and nuclear particles in the host cells was studied. Various factors including developmental changes of the macroschizont were demonstrated.

Attempts to infect normal bovine cells with schizonts *in vitro* using infected cells and freed parasites and to infect calves with separated schizont particles were unsuccessful.

Establishment of cell lines from normal bovine leucocytes and lymphoid cells using mitogens failed. The amounts of glucose uptaken and lactate produced in the culture of schizont-infected cells were measured and these correlated with cell growth. A surplus of glucose in growth medium did not affect its utilisation by the infected cells.

The methods for cryopreservation of *T. annulata*-infected cells were investigated. The cryoprotectants used, the methods of freezing and thawing, the duration of viability of the infected cells in low temperature storage and re-establishment of the cells after preservation at low temperatures were studied.

# CHAPTER 1

## INTRODUCTION

The first species of Theileria, T. parva was described by Koch (1898). Theileria annulata was found by Dschunkowsky and Luhs (1904) and described originally as Piroplasma annulata. Bettencourt, Franca and Borges (1907) compared the life cycle and morphology of Piroplasma bigemina (Smith and Kilborne 1893), with that of P. annulata and concluded that the latter should be placed in the genus Theileria. It was thereupon named Theileria annulata (Dschunkowsky and Luhs 1904), (Neitz 1957). The protozoans of this genus have presented difficulties in classification but the most up-to-date classification by Levine (1973) is as shown below.

| | |
|---|---|
| Phylum | Protozoa |
| Class | Piroplasmasida |
| Order | Piroplasmorida |
| Family | Theileriidae |
| Genus | Theileria |

The tick-borne, protozoal disease caused by T. annulata has been referred to as tropical piroplasmosis, tropical theileriosis, Egyptian fever and Mediterranean Coast fever, (Neitz 1957). The parasite is transmitted biologically by various species of the vector tick, Hyalomma, in nymphal and adult stages. The parasite is readily transmissible to susceptible animals, artificially, by inoculation of infective blood or organ suspensions, (Neitz 1957). Susceptible animals

are domestic cattle of all breeds and water buffalo (Bubalus bubalis Linn.), (Neitz 1957). Distribution of the disease, which is dependent on the presence of the vector tick, extends from North Africa and southern Europe, around the Mediterranean basin through the Middle East and south of U.S.S.R. and across India to the Far East. The life cycle of T. annulata in the vector tick and the host animal is similar to that of T. parva (Neitz 1957) but it has not been studied in as great detail as in T. parva. Sergent, Donatien, Parrot and Lestoquard (1936a, 1936b, 1936c and 1945) described the life cycle of T. annulata in the vector tick and bovine host. These authors believed that development of the parasite in the bovine host was by schizogony and in the vector ticks by sporogony. This is similar to the view of Cowdry and Ham (1932) for T. parva. This hypothesis has been disputed and the evidence available is not sufficient to confirm that a sexual cycle takes place in the tick, (Reichenow 1940). It is generally accepted that a small uninucleate body termed an "infective particle" is injected into the bovine host during feeding of the vector tick. There, then follows in the bovine host a pre-patent period during which the location of the parasite remains unknown until it appears some 10 to 14 days after tick attachment, in the local lymph node in the form known as the macroschizont. Multiplication occurs by schizogony, within the lymphocytes. The number of infected lymphocytes increases as they undergo mitosis. It appears as if the presence of the parasite accelerates the mitotic activity of lymphocytes. After a series of divisions as macroschizonts, microschizonts are produced within the lymphocytes. These break out of the host

cells and divide into individual micromerozoites which enter the red blood cells and assume the piroplasmic form which is the stage picked up by the tick. When ingested by the tick, feeding on the blood of an infected animal, the parasite enters a cycle which eventually produces a uninuclear body in the alveolar cells of the tick salivary gland. This remains dormant until the tick commences its next feed, when division takes place, producing, in a period of one to five days, a mass of infective particles which pass into the new bovine host during feeding. This is similar to the description for *T. parva* by Wilde (1967). Mortality in infected animals varies greatly according to the virulence of various strains of the parasite as well as the susceptibility of the cattle involved. The mortality rate is lower than in East Coast fever caused by *T. parva*. The latter produces a mortality of over 90 per cent, (Brocklesby, Barnett and Scott 1961; Wilde, Brown, Hulliger, Gall and MacLeod 1968). Mortality, due to *T. annulata* has been stated to be about 40 per cent in Palestine (Adler and Ellenbogen 1935), 20 to 40 per cent in Algeria (Donatien and Lestoquard 1938), Zero to 20 per cent among local breeds and 50 to 80 and even more than 90 per cent in exotic breeds in Iran (Rafyi, Maghami and Hooshman-Rad 1965), up to 76 per cent in India (Sen and Srinivasan 1937), up to 90 per cent in the enzootic regions in the U.S.S.R. (Yakimoff and Goussef 1936), and about 75 per cent in Bulgaria (Pavlov 1942). MacHatti (1935) concluded that at least 50 per cent of calves of Ayrshire cross-breed died due to theileriosis in Iraq, each year. Yousif (1969) reported that of 100 European cows imported into Iraq, 44 became infected with theileriosis and 29 died of the disease (Hooshmand-Rad 1973). It must be remembered in this

connection that in the areas where these mortalities have been recorded, many of the cattle are carriers so that the majority are exposed in very early calfhood when their resistance is high.

No drug so far has been proved conclusively to be effective against the disease, despite all attempts made, (Neitz 1957; Hawking 1958; Wilde 1967; Joyner and Brocklesby 1973; Hashemi-Fesharki and Shad-Del 1974; McHardy, Haigh and Dolan 1976).

Theileria annulata is, therefore, an organism of considerable importance in many areas where it acts as a major obstruction to the improvement of cattle production. Hitherto, attempts to ameliorate the problem have depended on control measures adopted against the tick vectors which have needed the use of acaricides and such management practices as zero grazing. These, however, are not readily effective and are economically expensive. Methods of artificial immunisation have, therefore, been sought. Recently work on the adaptation of T. annulata to tissue culture opened up the possibilities of producing an immunogen by attenuation of the parasite in vitro. Early work by Tsur (Tchernomoretz) (1945, 1947, 1953) showed that T. annulata-infected lymphocytes could be propagated in tissue culture. Subsequent work on T. annulata tissue culture by Brocklesby and Hawking (1958), Tsur and Adler (1962), Hulliger, Wilde, Brown and Turner (1964), Tsur and Adler (1965), Pipano and Tsur (1966), Hooshmand-Rad and Hashemi-Fesharki (1968), Hooshmand-Rad (1973 and 1975), Hashemi-Fesharki and Shad-Del (1973b) proved that T. annulata-infected lymphoid cells could be cultivated in explant plasma clots, on monolayers and in suspension culture. It was possible to attenuate the parasite in tissue culture which could be stored at -70°C and eventually be used as a vaccine against theileriosis,

producing partial immunity in the bovine animal.

Theileria parva-infected lymphoid cells also were cultivated in tissue culture, in a similar manner, though with more difficulty, (Tsur, Neitz and Pols 1957; Brocklesby and Hawking 1958; Hulliger et al 1964; Malmquist, Nyindo and Brown 1970; Moulton, Krausse and Malmquist 1971a and 1971b; Malmquist and Brown 1974). Danskin and Wilde (1976a and 1976b) succeeded in establishing various stages of T. parva from macroschizont to microschizont, micromerozoite and finally infection of bovine red blood cells, in vitro. If their success extends to achievement of infecting the vector ticks from the cultured, infected red blood cells, then a new field of investigation for studying the life cycle and development of the parasite is open. Considering the immense progress achieved in the study of theileriosis, with the aid of tissue culture one realises that any effort and speculation on every problem of the parasite in vitro is worthwhile. Brocklesby (1956) stated that if a standard procedure reproducing regular cultivation and growth of the parasite in tissue culture is achieved, "then the following applications appear feasible:-

1. The testing of potential schizonticidal drugs for in vitro activity against the parasite.
2. Close study of the morphology of the parasite.
3. Serial passage, maintenance and possibly attenuation of the organism.
4. Various experiments in immunity."

The importance of tissue culture in research on T. annulata has, therefore, been established. The work described in this thesis, based upon the development of readily reproducible

cultures of T. annulata was designed to investigate certain aspects of in vitro culture of the parasite which might contribute to knowledge of the parasite and its development.

# CHAPTER 2

## REVIEW OF LITERATURE

Isolation and subsequent cultivation in tissue culture.

For many years it has been known that tissue culture is a convenient method for the growth and study of various viruses, rickettsiae, and protozoa. The detailed study of any intracellular parasite is always handicapped by the difficulty of distinguishing between phenomena due to the parasite and those due to the host-cell. If the parasite can be grown outside its host-cell, the position is much simpler. Theileria spp. are strictly cell-associated so that growing the extra-cellular forms has not been, so far, possible. On the other hand no success in continuous cultivation of bovine leucocytes or lymphoid cells, in vitro, without any stimulation has been achieved. The use of such normal cells as the controls for the studies envisaged, including those on metabolism, is impossible. Any research in Theileria spp. in vitro must, therefore, be carried out in the Theileria-infected cells. It is generally accepted that for successful tissue culture certain environmental conditions and nutritive materials are necessary. These have been well defined by Paul (1975). Early work with Theileria annulata by Tsur (1945) showed that bovine serum alone would not support the multiplication of the parasite but provided for its survival. Subsequent work has shown that for survival and growth of cells infected with Theileria spp. a medium such as Eagle's supplemented with other nutrient materials is satisfactory. Eagle (1955a) proved seven vitamins to be essential for the survival and multiplication of

a mouse fibro blast (strain L) and a human carcinoma cell (strain He La) in tissue culture. Maximally concentrations of these vitamins:- cholin, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavine and thiamine were in the range $10^{-7}$ to $10^{-8}$g per ml. In another study, Eagle (1955b) described the necessity for twelve amino-acids in the L-configuration:- arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine to be included in a medium for growing mouse fibroblasts (strain L). He showed that seven other amino-acids:- alanine, aspartic acid, glutamic acid, glycine, hydroxyproline, proline and serine are non-essential. In the absence of any one of the essential amino-acids, there was no multiplication and the cells degenerated and eventually died. Eagle (1959) gave a list of the amino-acids required for survival and growth of every mammalian cell culture whether or not these are derived from normal or malignant tissue. This list is as stated in his paper (1955b) with the addition of L-glutamine. The author pointed out that none of the D-amino-acids substitute for the L-isomers, but most of the keto acids do indeed substitute for the corresponding amino-acids. Thus Eagle, discussing the metabolism of the essential amino-acids, states that glutamine is actively metabolised. Over and above its direct utilisation for protein synthesis, it is converted to glutamic acid, aspartic acid, asparagine and proline and to a limited degree to serine and alanine as well. Its carbon skeleton is heavily used in the biosynthesis of pyrimidines, presumably by way of aspartic acid. In the biosynthesis of both purines and pyrimidines, the glutamide amide nitrogen contributes more than half of the total base

nitrogen. It appears, therefore, necessary that L-glutamine must be included in a medium suitable for mammalian cell tissue culture.

From the introduction of tissue culture as a technique, success has been achieved in the propagation of viruses and some rickettsial pathogens. Early attempts to use this technique for the cultivation of parasitic protozoa achieved only minor success. In 1912, Bass and Johns attempted unsuccessfully, to cultivate Plasmodium spp. in vitro. Other workers have reported similar attempts which were also unsuccessful (Hawking 1945); (Gavrilov, Bobkoff and Lawrencin 1938; Henger and Wolfson 1939; Trager 1941 and 1943). In papers published in 1944, 1945 and 1951, Hawking, using a technique suggested by Jacoby (1944) for the cultivation of macrophages, was successful in growing Plasmodium gallinaceum in tissue cultures of chicken cells of the macrophage-reticulo-endothelial series. The increased use of tissue culture in the study of mammalian pathogens, particularly its successful application to the study of Plasmodium spp. encouraged work along similar lines with regard to species of Theileria. Tsur (Tchernomoretz) (1945) modified this method further to in vitro cultivation of Theileria annulata. This early success was achieved using the method described below. Some five to six square coverslips were attached to the bottom of a Carrel flask by a drop of plasma and chick embryo extract. A small fragment of infected calf spleen or lymph node was placed on each coverslip and calf plasma and chick embryo extract were applied to the tissue to be embedded. Three to five ml of a mixture of 30 to 40 per cent calf serum in Tyrode solution was added and the Carrel flask was tightly stoppered for incubation at 37°C or 38°C. In this system the author reported that macroschizonts

survived for at least twelve days but did not multiply. When he placed fragments of normal spleen in juxtaposition to the infected fragments at intervals of three to five days, the schizonts survived for 15 to 18 days but still there was no obvious multiplication. The addition of glutamine (3.0 µg per ml), pyridoxine (0.6 µg per ml), inositol (4.0 µg per ml) and riboflavine (0.04 µg per ml), to the mixture of serum and Tyrode solution induced multiplication of the schizonts which was observed in ten successive fragments of normal calf spleen during a period of two months. The author, therefore, concluded that multiplication of the schizonts was induced by growth factors (amino-acids and vitamins). In 1953, Tsur published a further report in which his description of the method and medium used were as in his previous papers. This appears to indicate that no major progress had been made in the intervening period. Brocklesby (1956) reported, attempts to cultivate T. parva in tissue culture, using nine media, composed of various proportions of chick embryo extract, Earle's saline, Hank's saline, bovine amniotic fluid, bovine serum, pig serum and bovine lymph node extract with and without penicillin/streptomycin. One of the media was the same as that which Tsur (1945) had used for T. annulata tissue culture, but none of them apparently contained amino-acids and vitamins. The tissues used for the culture were lymph node, spleen and bone-marrow from T. parva-infected animals prepared both by biopsy at various stages of the disease and after slaughter. The method of cultivation was the same as that of Tsur used for T. annulata; the clot tissue explant on coverslips in a large Carrel flask and Petri dish being nourished with fluid media. Brocklesby tried to cultivate T. parva by two methods. The first was under

anaerobic conditions, as he found that oxygen/carbon dioxide tension so produced was favourable to cell growth. The second was with trypsinised monolayer cultures both of infected and uninfected lymph node and spleen. The author reported that, during 24 to 48 hours only migration of cells took place. Healthy lymphocytes rarely survived for more than four days. After 48 hours, fibroblast activity began until most of the coverslips were supporting a meshwork of fibroblasts with a few macrophages and lymphocytes. T. parva schizonts survived for only a short period and did not multiply. The author concluded that growth of T. parva in tissue culture depends on the survival of its host-cell. Tsur (Tchernomoretz), Neitz and Pols (1957) achieved multiplication of T. parva schizonts in tissue culture for a period of ten days. Thereafter, the schizonts were maintained without multiplication for a further five days. The authors showed that the addition of penicillin, streptomycin, aureomycin and plasmoquin to the cultures did not affect the multiplication of Theileria-infected cells but that these could persist for 20 days in the cultures. The medium used was 35 per cent bovine serum in Tyrode solution with the addition of glutamine (3.0 µg per ml), pyridoxine (0.6 µg per ml), inositol (4.0 µg per ml) and riboflavine (0.04 µg per ml). The pH often ranged from 7.5 to 9.0, but was initially adjusted to 7.5 in every trial. Brocklesby and Hawking (1958) reported the cultivation of T. annulata and T. parva in tissue culture. T. annulata was isolated by implantation of infected spleen, lymph node or liver and was grown in vitro for over 59 days. Multiplication of the parasites occurred clearly and the cultures were infective for cattle when tested after 17 and 42 days.

T. parva was maintained in tissue culture for 14 days, with some multiplication during the first few days. T. parva appeared to exert a toxic influence over the adjacent cells; and if the initial implant of spleen or lymph node contained many parasites, growth of all types of cell was inhibited. The media used by the above authors were: (i) Forty per cent bovine serum, five to ten per cent chick embryo extract and 50 to 55 per cent Hank's salts together with the supplements recommended by Tsur, (glutamine, pyridoxine, pyridoxal, riboflavine and inositol). (ii) Medium 199, an enriched medium described by Morgan, Morton and Parker (1950) to which was added five per cent sodium bicarbonate. Hawking (1958) grew T. annulata-infected lymph node, spleen and liver tissues by explant techniques for the study of chemotherapeutic screening. The media and techniques were similar to those described by Tsur (1945) and Brocklesby and Hawking (1958). He used Hank's and Earle's salts and found both equally good. Tsur's medium and medium 199, both supplemented with 40 per cent calf serum, 2.5 per cent chick embryo extract and a small amount of sodium bicarbonate, were compared and the author stated that although growth of cells and of parasites was somewhat irregular, there was no constant difference between the two media. The schizont-infected cells from lymph nodes gave the best growth. Tsur and Pipano (1959) cultivated T. annulata-infected spleen tissue fragments on coverslips in a clot of bovine plasma and chick embryo extract in spleen tissue cultures of rodents, especially Meriones trystrami shawii and the laboratory white mouse. They obtained satisfactory growth and multiplication of the parasite in six passages for more than two months. Tsur and Adler (1962) described a method of monolayer tissue culture

of bovine tissues infected with T. annulata which were minced and trypsinised and resuspended in Earle's solution containing bovine serum, antibiotics and yeast. The cultures were incubated at 38°C and the supernatant fluids of the tissue cultures were replaced every three to four days with fresh Earle's solution. The propagation of tissue culture, for three months, up to the date of the report, was successfully continued. The authors added normal bovine cells for about two months then continued passage of the culture without the addition of normal cells and showed that the schizonts could multiply without these cells. Tsur, Adler, Pipano and Senft (1964) confirmed the method of monolayer and mixed culture of T. annulata-infected tissue. The authors subcultured successfully and continuously both the cells in suspension and resuspended cell layers. They stated that the tissue culture had been growing for two years. They did not contribute any information on growth medium, nor did they indicate a realisation of the advantage of suspension cultures. Hulliger et al (1964) employed baby hamster kidney (BHK) cells successfully as a feeder layer for cultivating T. annulata and T. parva in vitro. Bovine lymphatic tissues infected with Theileria spp. were satisfactorily used in isolation and cultivation. Hulliger (1965) reported isolation and cultivation of T. annulata, T. parva and T. lawrencei from various schizont-infected tissues such as: spleen, lymph node, buffy coat from the peripheral blood, liver, kidney and thoracic duct lymph. These tissues were cultured in association with BHK cells. The author stated that BHK feeder layer, by rapid growth, provides favourable metabolic conditions for the schizont-infected cells during the early stages of establishment; it also helps these cells to attach to

the surface, where multiplication is favoured. The author reported that the most successful isolations were made from those materials prepared from experimental animals when they were in early and middle stages of infection with the parasite. She pointed out that _T. parva_ was more difficult to isolate and cultivate than the two other species, as only five out of 56 trials were successful with _T. parva_. Isolation of schizont-infected cells from the buffy coat resulted in success with _T. annulata_ and _T. lawrencei_ but not with _T. parva_. The growth medium used was Earle's with ten per cent calf serum supplemented with ten per cent of a 2.0% stock solution of tryptose phosphate broth and antibiotics. Tsur and Adler (1965) grew _T. annulata_ from the peripheral blood of a bovine animal acutely ill with theileriosis, by the monolayer method. The medium used was Earle's solution with calf serum and antibiotics. The buffy coat was prepared from heparinised blood by the addition of chick embryo extract and incubated at 37°C for three hours. Then, the coagulated buffy coat was washed in Earle's solution and trypsinised in a 0.25 per cent trypsin solution for a few hours. This trypsinised cell suspension was mixed with bovine serum, centrifuged and the deposit was washed again and resuspended in Earle's solution. The cells finally were cultured at the rate of $2.0 \times 10^6$ to $3.0 \times 10^6$ per ml at a pH of 7.2, in flasks having coverslips attached to the floor. The authors carried out subculturing every three to four days, removing the supernatant fluid and trypsinising the cell layers. Tsur and Pipano (1966) continued the cultivation of _T. annulata_-infected tissues in association with monolayers. The primary culture was maintained for nine months with bi-weekly changes of the medium and sub-cultures were made every one or two months.

The authors stated that a strain of T. annulata which was maintained in tissue culture for 20 months continuously, had been completely attenuated and resulted in an ideal vaccinal strain. They claimed that this strain produced immunity in inoculated animals without causing any thermal or parasitic reactions. Pipano and Tsur (1966) showed attenuation in various strains of T. annulata, already maintained in tissue culture for three to 35 months. The schizont-infected cells grown in vitro were found infective when injected into susceptible cattle. Zablotskyi (1966 and 1967) reported the cultivation of T. annulata schizonts in tissue cultures derived from infected lymph nodes and spleen. The growth media used were Eagle's medium and medium 199. The authors claimed to have demonstrated the attenuation in virulence of the parasite "in the surprisingly short periods of" eight, nine and 23 days; and immunity was produced in the inoculated cattle with tissue culture material. Hooshmand-Rad and Hashemi-Fesharki (1968) succeeded in growing schizont-infected lymphoid cells in suspension, independent from any cell association and without any mechanical means. These authors compared cultivation of three strains of T. annulata, with different virulences. They cultured a very mild strain (S.11) with ease and a virulent strain (S.15) with some difficulty at first, but failed to culture a very virulent strain (S.3). The authors, hence, concluded that there was an effect of virulence on cultivation of T. annulata in vitro. They pointed out finally that cultivation of the very virulent strains of T. annulata such as S.3, is very difficult, if not impossible. The authors used Eagle's medium with ten per cent calf serum. In several trials to cultivate S.3, they tried "different media such as YLH-YLE-Eagle" with ten to 40 per cent

calf serum without success being achieved. These authors used biopsy samples of schizont-infected tissue of lymph node, spleen and liver which were washed, centrifuged and dispersed in the medium and cultured in small glass bottles, without trypsinisation. The cultures which were initiated with 1.0 x $10^5$ cells per ml of medium were passaged every four days. Malmquist et al (1970) established spleen cell lines from calves experimentally infected with T. parva. The authors observed that the parasitised cells disappeared for about five to six weeks in the cultures. Then, schizont-infected lymphoblasts, which could readily be sub-cultured without feeder layers emerged. The authors pointed out that plating of infected cells was greatly enhanced by culturing the cells on preformed monolayers. The medium used was Eagle's MEM with Earle's salts supplemented with 20 per cent foetal calf serum and 0.1g per litre L-$\beta$-asparagine. Infected cell cultures were prepared according to Malmquist, Van der Maaten and Boothe (1969) for the study of bovine leucosis. Cell deposit from supernatant fluid of the culture, as well as dispersed monolayer culture with 0.02 per cent ethylene-diamine-tetra-acetic acid, (EDTA) were used for continuous passage, both with success. Moulton et al (1971a) cultivated schizont-infected spleen cells from a T. parva-infected calf, in Eagle's MEM plus 20 per cent foetal calf serum and antibiotics. The method of isolation was the same as that used by Malmquist et al (1970), starting with large clumps of tissue and approximately 5.0 x $10^6$ cells per ml suspension. Sub-cultures were made twice a week in plastic Falcon flasks, at 37°C; and finally the cells showed nearly 100 per cent infection with schizonts. Viability tests were done using erythrosin B and the cells were counted. Moulton et al (1971b) in

another paper described isolation and cultivation of spleen cells of cattle experimentally and naturally infected with T. parva. Conditions of cultivation were the same as in their previous publication (1971a). They measured the growth rate of the schizont-infected lymphoblasts in suspension culture and found that in the course of five days six fold growth was obtained. The culture initially was seeded with 4.0 x $10^5$ cells per ml and over a period of four days there was a sigmoid growth, then on the fifth day the growth started to decline. Hooshmand-Rad (1973 and 1975) described isolation and cultivation of T. annulata in Eagle's medium (1955) with some modifications in amino-acids and vitamins. This medium contained ten per cent calf serum and was supplemented with 1.0g lactalbumin hydrolysate and 0.2g yeast extract per litre. Hooshmand-Rad stated that the addition of ten per cent calf serum to the medium was quite adequate and also there was no adverse effect on propagation of schizont-infected cells when calf serum was substituted by sheep serum or horse serum. He demonstrated that the addition of lactalbumin hydrolysate and yeast extract improved the growth of the infected cells up to more than twice as much growth as with the Eagle's medium alone. Hooshmand-Rad concluded from his experiments that isolation of T. annulata-infected cells in tissue culture was merely a transplantation and not a transformation. Stagg, Brown, Crawford, Kanhai and Young (1974) succeeded in isolating lymphoblastoid cell lines infected with T. lawrencei from leucocytes of a buffalo. The leucocytes obtained from heparinised blood were cultured on a bovine embryo spleen, (BESP) feeder layer. The authors stated that after 18 to 42 days transformation occurred in a manner similar to that which occurred with T. parva, (Malmquist et al 1970) and

the infected lymphoblastoid cells became detached from the monolayer. Leucocyte cultures in the authors' trials, without a BESP feeder layer, failed to transform. It is understood that this is the first recorded case of cultivation of T. lawrencei macroschizonts in buffalo lymphoid cells. The growth medium used was Eagle's MEM, with Earle's salts supplemented with 0.1g per litre L-$\beta$- asparagine and 20 per cent FCS with antibiotics. This medium was further supplemented, immediately before use, with 0.292g per litre L -glutamine. The heparinised blood, in 20 ml aliquots, was left in Universal bottles at $25^{o}$C for an hour to sediment; then ten ml of leucocyte-rich plasma were collected from each 20 ml of blood. The infected leucocytes were separated from the plasma by five minutes centrifugation at 180 G. Resuspended leucocytes in the culture were placed on a BESP monolayer in a plastic T-flask and incubated at $37^{o}$C. According to these authors, a greater amount of L-glutamine seemed favourable to the growth of cells. Malmquist and Brown (1974) established lymphoid cell lines infected with T. parva from lymph node biopsy samples of five cattle reacting to ECF. In four out of the five cases, the infected lymphoid cells grew successfully on a BESP monolayer. One cell line, infected with T. parva was, however, established without the aid of a feeder layer. Growth medium and other conditions of cultivation used were the same as described previously by Malmquist et al (1970).

Morphological changes in the development of the stages of Theileria spp.

The life cycle of the theilerias is incompletely known, as infection of the bovine host by inoculation of infective particles from a feeding tick is first manifested in the host several days

after the inoculation. The first sign of the parasite, at this time, which can be seen by bright light microscopy, is a small macroschizont in a cell of the lymphatic series assuming blastoid characteristics. What has happened to the infective particle between the time of its injection to the first appearance of a small macroschizont is not yet known. The macroschizont and its development in the bovine host has been well described for T. parva by many authors, (Neitz 1957; Jarret and Brocklesby 1966; Barnett and Brocklesby 1966a and 1966b; Brocklesby and Barnett 1966a and 1966b; Wilde 1966; Jarret, Crighton and Pirie 1969; and Brocklesby 1970). Sergent et al (1936a, 1936b, 1936c and 1945) have described the changes in the parasite, T. annulata, in the bovine host. Tissue culture of theilerial species has hitherto been uniformly successful only with the macroschizont stage. In the bovine host, after a period of replication of the nuclear parasites of the schizont and a corresponding increase in the cytoplasm of the host cells, there is a transformation of some of the parasites to a microschizont form in which the nuclear particles become more numerous, more compact and more uniformly coccal in shape. This microschizont appears to burst out of its host cell by disruption of the cell cytoplasm and breaks up into what are described as micromerozoites. These are particles of nucleoplasm invested with a thin layer of cytoplasm and they appear to be responsible for entering the bovine erythrocytes and assuming the piroplasmic form. These stages have been clearly described and illustrated by several authors (Reichenow 1940; DeKoch 1957 and Wilde 1967).

In studies on the culture of lymphoblasts derived from the infected host of T. parva, T. annulata and T. lawrencei, it has been shown that the infected lymphoblast appears to be stimulated into

mitosis by the presence of the parasite while non-infected cells die out. Thus, cultures of transformed lymphoblasts contain almost exclusively infected cells. The reason for this became apparent when Hulliger _et al_ (1964) described the process of division of infected cells in tissue culture, showing that when the host cell entered the process of mitosis, the macroschizont became involved by associating with the chromosomes at prophase and being aligned with them in the central plate at metaphase. In the subsequent separation of the chromosomes, the parasite appears to be subjected to tension along the spindle so that at telophase it is extended between the two groups of chromosomes and breaks into two roughly equal parts which, at reconstitution, form macroschizonts in each of the new daughter cells. Wilde (1967) describes how, having discovered this process, he and his colleagues looked for, and found, the same characteristic figures _in vivo_ in cattle infected with _T. parva_. It was thus apparent that the macroschizonts could propagate, having become established in the host lymphoblast while still remaining invested in host cytoplasm. Until this discovery it had been generally accepted that the macroschizont broke out of the host lymphocyte producing macromerozoites which, in turn, entered fresh lymphocytes to continue the macroschizont cycle. Reichenow (1940 and 1941) had cast doubt on this and suggested a possible close relationship between the division of the parasite and mitosis of the host cell. Brocklesby and Hawking (1958) had remarked the lack of evidence for the infection of new lymphocytes by macromerozoites and the work of Hulliger _et al_ (1964) caused the existence of this process into question. These authors, however, stated that while a method of

propagation of the parasite T. parva which ensured its continual investment in its macrophage stage in host cytoplasm had been demonstrated, they could not say whether or not any other means of propagation could occur, as for example, by the production of macromerozoites. Hulliger, Brown and Wilde (1966) demonstrated that in long term cultivation of T. parva-infected lymphoid cells in association with BHK, particles of Theileria divide at about the same rate as the host cells, so that the average number of these particles per cell remain within a fairly constant range. In these established cultures at 37°C, the macroschizonts continued to multiply along with the host cell mitoses, without forming any microschizonts. Thus, only the first visible stage of the parasite was produced in tissue culture. In an attempt to produce microschizonts the authors incubated the cultures at 40°C and succeeded in producing a few microschizonts in most cultures. Then several groups of cultures were incubated at 41, 42 and 42.4°C for various periods of time and microschizonts and transitional forms appeared in many of the schizont-infected cells, (28 to 36 per cent). The authors explained that high temperatures appeared to slow down multiplication of the host cells but not the rapid replication of the macroschizonts and eventually the excess of parasite nuclear particles caused transformation to the microschizont form and the parasite were freed, each in a separate cytoplasmic envelope as micromerozoites. Thus a morphological change from parasite bodies containing a comparatively small number of large, spur-like, or triangular shaped nuclear particles to the small, round nuclear form of microschizont as seen in vivo was brought about. The authors were unable to cause this change by other means attempted such as the use of partially deficient

medium, varied oxygen tension or low temperatures which could also slow down the growth of the host cells.

Until this time, the only form of the parasite reported in tissue culture had been the macroschizont. Danskin and Wilde (1976a and 1976b) succeeded in stimulating T. parva-infected lymphoid cells to produce microschizonts and further developmental stages. The authors used Eagle's MEM with Earle's salts supplemented with ten per cent FCS and 0.1 per cent extra L-β-asparagine for the routine cultivation of macroschizont-infected lymphoid cells as described by Malmquist et al (1970). When five to ten per cent bovine lymph was added to the medium and the amount of FCS was increased to 20 to 30 per cent, the macroschizont-infected cells cultured in this medium divided rapidly and macroschizonts changed into microschizonts and then into micro-merozoites. When washed bovine red blood cells were added to the culture, some of them were infected with micromerozoites which were seen as piroplasms.

The authors attributed this production of microschizonts, micromerozoites and piroplasms to the presence of the bovine lymph and the increased amount of FCS in the tissue culture medium. They found the addition of the bovine lymph without FCS appeared to be toxic to the infected cells. The piroplasms appeared in the rbc from six hours to 54 hours after the red cells were added to the cultures. The number of red cells infected with piroplasms was low and was estimated as about 0.1 per cent, but they suggested that further experiments might bring about a higher rate of infection. This limited success has opened a new field of study of the life cycle of T. parva, so that if infection of the vector tick can be achieved from such cultures, study of the whole life

cycle of this parasite may become possible without the necessity for the use of expensive experimental animals. In none of the reported work on *T. annulata* have these changes been described.

Transference of schizonts to normal uninfected cells.

As shown above, the method of propagation of the macroschizonts of *T. parva* synchronously with the mitosis of the host cells has been established. The question as to whether the alternative method by the production of macromerozoites and the infection of healthy cells occurs, remains to be answered. This gap in our knowledge was emphasised at a conference held in Nairobi in December 1974 on "East Coast fever research". (Wilde 1975, Personal Communication). Brocklesby (1956), after attempting to grow *T. parva*-infected lymphoid cells, showed that macroschizonts did not invade uninfected fibroblasts or macrophages which were available in tissue explant cultures.

Brocklesby and Hawking (1958) in their attempts to cultivate *T. annulata* and *T. parva* in explant culture form, tried to transmit the schizont infection to uninfected bovine spleen, liver and embryo kidney cells which were growing in monolayers, but no success was achieved. Hulliger *et al* (1964) scanned many thousands of biopsy smears prepared from lymph nodes of *T. parva*-infected cattle. They did not find any evidence indicating infection of uninfected cells by macromerozoites. These authors in the established cultures of lymphoid cells infected with *T. annulata* and *T. parva* in association with BHK cells, also observed neither release of single merozoites nor infection of uninfected cells by these particles. Hulliger (1965) confirmed the previous report and indicated that theilerial particles never infected BHK cells

nor other uninfected cells. She observed that bovine uninfected cells did not undergo mitosis without multiplying schizonts in them. Pipano (1965), in a review of Theileria, reported the free schizonts to have appeared in culture but no penetration of these free schizonts into the normal cells was observed. Wilde, Hulliger and Brown (1966) injected healthy female cattle with schizont-infected male cells from culture and also a healthy male animal was inoculated with cultures of schizont-infected cells of female origin. In both cases infected cells were re-isolated and established in tissue culture. The recovered cells were studied by chromosome analysis and it was shown that in each case "the parasite was transferred from the inoculated tissue culture cell to a cell of the bovine recipient". This was explained by the authors as possibly due to phagocytosis. Malmquist et al (1970) attempted unsuccessfully to infect a bovine embryo spleen (BESP) monolayer of female origin with a culture of T. parva-infected male lymphoid cells, but no success was achieved. The author also tried to separate the schizonts by filtering the suspension cultures through 0.3 μm and 0.6 μm membrane filters and centrifugation, which were then mixed with the BESP culture. The chromosome analysis showed no new-infection in normal cells in vitro. The author, therefore, concluded that neither by membrane filtration nor by centrifugation, were the schizonts separable from the host cells. Moulton et al (1971a) indicated that transference of extra cellular form of T. parva, in vitro from cell to cell was not observed. The author stated that this new-infection might not occur in lymphoblasts in vivo either. Stagg et al (1974) successfully established cell lines of T. lawrencei-infected lymphoblasts originating from the buffalo in association with a

female BESP. They then made karyotypic analysis and found that established infected cells were of buffalo origin and, therefore, it was understood that no transmission of the schizonts of T. lawrencei into the normal bovine cells, BESP took place. Malmquist and Brown (1974) cultivated T. parva-infected lymphoblasts, derived from the lymph node of an infected male animal, over a female BESP. The authors reported that chromosomal analysis showed that 100 per cent of the cells infected with the schizonts were male, and no schizont infection occurred in the BESP cells. Irvin, Brown, Boarer, Crawford and Kanhai (1974) studied cell fusion in cultures of T. parva and T. lawrencei by a labelling method. According to these authors, in some samples, labelled macroschizonts were found in cells with unlabelled nuclei. This could be explained in one of three ways:

(a) The uptake of label by the macroschizonts was independent of any uptake by the cell nucleus.

(b) The macroschizonts could be phagocytosed by other cells.

(c) Cell fusion of labelled and unlabelled cells had taken place resulting in the transfer of the parasite from one cell to another.

These authors finally pointed out that "this propensity of Theileria-infected cells to fuse may be a means whereby parasites can be transferred to uninfected cell".

Stagg, Kanhai, Young and Brown (1976) established cell lines infected with a Theileria sp. or Cytauxzoon sp. from a male eland on monolayers of a female BESP, and made karyotypic analysis. They found schizont particles in the male eland cells only.

Glucose uptake and lactate production.

There is no information on glucose uptake and lactate production by T. annulata-infected cells in tissue culture. The reason seems to be that extra-cellular macroschizonts never survive and grow in tissue culture independent from the host cell, thus, any metabolic study on the parasite must be carried out on the combination of the parasite and the host cell as an unseparable living identity. To do this, a control which is a continuous suspension culture of normal bovine lymphocytes growing without any stimulatory factor is necessary; and this has not been achieved, as yet. There are many reports on growing bovine lymphocytes in vitro, but being stimulated by mitogens. This conditional growth of normal cattle lymphocytes involves in (a) separation of normal lymphocytes and (b) stimulation of these to mitose.

(a) There are many methods to separate leucocytes from peripheral blood and lymphoid cells from tissues. Various methods are based on any of these three ways:- (1) To utilise the property of cell adhesion to the surface of culture vessel. (2) Lysing erythrocytes and separating WBC. (3) The technique of gradient centrifugation, using different materials either to hasten sedimentation of erythrocytes or to separate different types of cells, which possess different specific densities, in several layers. This separation is the matter of choice which may depend upon the following factors:- purity, yield, simplicity of technique and donor species (Weir 1974). Tokuda, Fukusho, Morimoto and Watenabe (1962) separated leucocytes from heparinised or defibrinated blood of healthy cattle by utilising the property of leucocytes' adhesion to the surface of glass. The authors incubated

the whole blood mixed with antibiotics in test tubes in slope position at 37$^{o}$C for a few days. They then washed off the suspension cells and added growth medium to the remaining adhered WBC, which were then used in studies on rinderpest virus. Odajima and Sonoda (1970) separated bovine leucocytes from peripheral blood by lysing the erythrocytes using distilled water. The same authors (1971) used ammonium chloride solution, lysed erythrocytes and separated normal bovine lymphocytes. Carlson and Kaneko (1973), separated leucocytes from bovine peripheral blood using sodium chloride solution in PBS to lyse erythrocytes.

Joel, Adamik, Chanana, Cronkite, Schiffer and Sipe (1969) separated lymphocytes from normal calves and goats by means of liquid silicone. The authors used gradient centrifugation in this work.

Nichols, Levan and Lawrence (1962) used bovine fibrinogen to accomplish sedimentation and separation of erythrocytes, and thereby separated bovine WBC for chromosome study. Biggers and McFeely (1963) separated bovine WBC by using Ficoll solution. They believed that this gave more satisfacory results than other materials like dextran, bovine fibrinogen or bovine albumin. Sodium metrizoate/Ficoll solution (Lymphoprep) has been used successfully for separation of WBC and also various types of blood cells. The density of this solution is described as to be 1.077 $\pm$ 0.001 g per ml. This solution and other similar components with different commercial names have been employed for the above purpose by various authors:- (Böyum 1964 and 1968; Favour 1964; Harris and Ukaejiofo 1969; Thorsby and Bratlie 1970; Ting and Morris 1971).

Separation of lymphoid cells from tissues such as lymph

node and spleen is a routine work of laboratories as described by many authors including Weir (1974) and Paul (1975).

(b) Stimulation of normal bovine lymphocytes can be achieved by various mitogens. Phytohaemagglutinin (PHA) has been vastly used to stimulate cells from various animals including cattle. Normal bovine blood leucocytes respond to this stimulator (Ling and Kay 1975 and others), but the cells undergo mitosis only in a short term, a few days period. Many authors have taken advantage of this temporary activation of the cells to study bovine chromosomes.

Cryopreservation and retrieval of schizont-infected cells.

Cryopreservation of parasitic protozoa came to practice by Laveran and Mesnil (1904) who exposed trypanosomes for 15 minutes to -191°C in liquid air, the parasites "although after this short period storage" survived and retained their virulence. Coggeshall (1939) showed that Plasmodium-infected monkey blood preserved and thawed in rapid method was infective after 70 days storage (Taylor 1975). Occasional observation by Polge, Smith and Parkes (1949) on protective property of glycerol for avian spermatozoa appeared as an outstanding discovery in cryobiology. Since then glycerol has ever been used in cryopreservation of various types of cells. Polge and Soltys (1957) examined various concentrations of glycerol in different methods of slow and rapid cooling for preservation of *Trypanosoma brucei*, *T. congolense* and *T. rhodesiense*. Lovelock and Bishop (1959) showed that dimethyl sulphoxide (DMSO) protected erythrocytes and spermatozoa during freezing and thawing; and thereafter this cryoprotectant has been used in routine preservation of various organisms including

parasitic protozoa. Diamond (1964) reviewed cryopreservation of a fairly large number of protozoa (from infected culture and blood) using glycerol and DMSO at various concentrations. Cunningham, Lumsden and Webber (1963) preserved trypanosome-infected blood in capillary lymph tubes by the slow method, using glycerol at a final concentration of 7.5 per cent, as cryoprotectant. The authors adopted this method for low temperature storage of other protozoan genera, including Trypanosoma, Plasmodium, Leishmania, Trichomonas, Babesia and Theileria. Tsur and Pipano (1962) stored strains of T. annulata in bovine tissues at -70°C in sealed, four to ten ml ampoules, for five months. The authors used 15 per cent glycerol as cryoprotectant and froze the samples by slow method down to - 50°C and then transferred into storage at -70°C. Theileria-infected materials were blood and ground-up suspension of spleen and liver in buffered saline. These were proved to have remained viable and infective for up to five months when samples of them were thawed and inoculated into susceptible cattle. Tsur et al (1964) reported briefly that they preserved schizont-infected lymphoid cells obtained from suspension and layer culture at -70°C using ten to 15 per cent glycerol as cryoprotectant. Hulliger (1965) preserved schizont-infected cells from cultures, using ten per cent glycerol at -79°C. The infected materials which were of T. annulata, T. parva and T. lawrencei were cooled by a slow method taking two to four hours. These stabilates were kept frozen for several months and at intervals were thawed and used for culture. Rafyi, Maghami and Hooshmand-Rad (1967) kept T. annulata-infected tissues at -70°C for up to 277 days. They used as cryoprotectant twelve per cent glycerol for infected citrated blood and 15 per cent glycerol for infected liver, spleen

and lymph nodes. The materials were dispensed into 20 ml ampoules and 50 ml flasks and were cooled by the slow method. The authors proved the infectivity of these materials by inoculating samples of them at different intervals into susceptible calves. Hashemi-Fesharki and Shad-Del (1973a) maintained bovine lymphoid cells infected with various strains of T. annulata at -70°C for up to 1095 days. The materials were schizont-infected cell culture suspensions, citrated blood and ground-up tissues such as spleen, liver and lymph nodes in PBS. Ten per cent glycerol for tissue culture materials and 13.5 per cent glycerol for emulsified tissues and blood were used as the cryoprotectant. The samples which were kept directly at -70°C subsequently were re-cultured and inoculated into susceptible calves and proved to have been viable and infective. The same authors (1973b) in the course of vaccination of cattle with anti-theileriosis vaccine, successfully used the schizont-infected cells which were stored at -70°C by the same method, after various periods of storage. Hooshmand-Rad (1973) preserved schizont-infected cells in 20 ml bottles, using ten per cent DMSO and 7.5, 10.0 and 15.0 per cent glycerol as the cryoprotectants. The author froze down the infected cells in two ways:-

(a) The material was cooled one °C per minute down to -30°C, then it was transferred to storage at -70°C.

(b) Slow uncontrolled method in which the cell container was transferred directly into a deep freeze at -70°C.

This author concluded that:- 7.5 per cent glycerol was the optimal concentration compared with other percentages, 10.0 per cent DMSO was more suitable than 7.5 per cent glycerol, and the slow uncontrolled method was preferable to the slow controlled method.

He stated that temperatures of 26 to 35°C, 4 to 8°C and -22°C were not suitable for storage of infected lymphoid cells. Cunningham, Brown, Burridge and Purnell (1973) preserved infective particles of T. parva in a mixture of glycerol and precolostral calf serum (PCS) containing 7.5 per cent final concentration of glycerol. This was wrapped in cotton-wool and placed at -80°C in slow uncontrolled method. The authors thawed the stabilates rapidly in a water bath at 37°C. These authors stated that ground tick supernates infected with T. parva preserved at either -80°C or -196°C, retained their viability for at least a year.

Theileria-infected stabilates have generally been reported to have been thawed rapidly at 37°C to 40°C, but few much details have been contributed on the retrieval of schizont-infected cells. Hulliger (1965) stated that schizont-infected cells after preservation at -79°C were defrosted rapidly, centrifuged immediately and resuspended in growth medium. Hooshmand-Rad (1973) thawed the stabilate rapidly but added growth medium to the deposited cells slowly in order to cause slow elution of cryoprotectant.

# CHAPTER 3

## MATERIALS AND METHODS

### 1. Strains of Theileria annulata.

Strain 3 (S.3) was isolated from a bovine animal in Iran at the Razi Institute and maintained by blood passage in cattle for several years. This strain was transferred to tissue culture by the present author. S.3 is very virulent but does not produce erythrocytic forms.

Strain 15 (S.15) was isolated from a calf in Iran by Hooshmand-Rad and Hashemi-Fesharki (1968). This is a moderately virulent strain and does not produce erythrocytic forms.

Strains (S.19), (S.20) and (S.21) were isolated from naturally infected cattle and adapted to tissue culture by the present author from blood and organ tissues of calves artificially infected by blood stabilates. These strains are virulent and produce erythrocytic forms in the bovine host.

The strains of T. annulata S.3 and S.15 were used throughout this study. In Chapter 4 strains S.19, S.20 and S.21 were used in addition.

### 2. Theileria parva.

The Muguga strain of T. parva was obtained from the FAO/UNDP team working in EAVRO Muguga. This strain originated from a male Bos taurus animal E174 in September 1971, at Muguga.

### 3. Experimental animals.

Male Holstein calves aged five to 15 months were used for isolation and production of blood stabilates. These animals were

maintained in tick-free stables.

Uninfected adult Ayrshire cattle were used as the source of normal leucocytes and red blood cells (rbc).

Healthy sheep were used as the source of normal sheep leucocytes and rbc.

Normal rabbits were used for preparation of rabbit-anti bovine lymphocyte antiserum (RABLS), normal rabbit serum and rabbit leucocytes.

4. Growth media and supplements.

The growth media used in this study were:-

(a) Eagle's medium with Hank's salts. This was based on Eagle's medium (Eagle 1955) with ten per cent neo-natal calf serum (CS) and lactalbumin hydrolysate and yeast extract (LY) as modified and described by Hooshmand-Rad (1973).

(b) Eagle's minimum essential medium (MEM) with Hank's salt base (Eagle 1959)[1] supplemented with CS and LY as for (a) above.

(c) Eagle's MEM with Earle's balanced salt solution (BSS)[1] with CS and LY as in (a) and (b) above.

(d) Tissue culture medium 199 (TC 199)[2] with CS and LY.

(e) Hank's balanced salt solution (BSS) with LY (lactalbumin hydrolysate increased to five g per litre and yeast extract increased to one g per litre), supplemented with ten per cent CS.

(f) Earle's BSS supplemented as in (e) above.

(g) Phosphate buffered saline (PBS) without calcium and magnesium chloride (Dulbecco 'A') supplemented as in (e) and (f) above.

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(1) Flow Laboratories Ltd., Irvine, Scotland.

(2) Wellcome Reagents Ltd., Beckenham, England.

To all media were added, penicillin ($10^4$ U. per 100 ml) and streptomycin ($10^4$ µg per 100 ml) and the pH was adjusted appropriately.

The glutamine component in all media was added separately just prior to use after being sterilised by filtration.

Growth supplements:-

(i) Calf serum (CS)[1]

(ii) Foetal calf serum (FCS)[1]

(iii) Non-essential amino-acids (NEAA)[1]

(iv) Lactalbumin hydrolysate and yeast extract (LY)[2]

The LY was prepared in stock solution by dissolving 25g of lactalbumin hydrolysate and five g of yeast extract separately in deionised distilled water (DDW) at approximately 70°C. These were combined and made up to a total volume of one litre and sterilised by filtration or autoclaving (10 minutes at 10 lb. per sq.inch). This was preserved at -24°C in aliquots in Universal bottles.

(v) Tryptose phosphate broth (TPB) was made up in stock solution as two per cent w/v as follows:- 2.0g tryptose, 0.2g dextrose, 0.5g sodium chloride and 0.25g disodium hydrogen phosphate in 100 ml DDW. The solution was sterilised in the autoclave at 15 lb. per sq. inch for 15 minutes.

(vi) L-$\beta$-asparagine[3] was made up in a x 100 strength stock solution of ten mg per ml in DDW and sterilised by filtration.

(vii) L-glutamine, a basic constituent of all media (a x 100 strength) was made up separately by dissolving 2.92 g L-glutamine[3] in 100 ml DDW. This was sterilised by filtration and then stored

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(1) Flow Laboratories Ltd.

(2) Difco Laboratories Ltd., East Molesey, Surrey.

(3) BDH Chemicals Ltd., Poole, England.

at $-24^{\circ}C$. One ml (29.2mg) of this solution was added to 100 ml of growth medium prior to use.

(viii) D-glucose was made up as a ten per cent solution in DDW, sterilised by filtration and stored at $+4^{\circ}C$.

(ix) Antibiotics.

A stock solution of sodium benzyl-penicillin[1] and streptomycin sulphate[1] (PS) was made up in DDW to contain $5 \times 10^4$ U. penicillin and $5 \times 10^4$ µg streptomycin per ml. This solution was sterilised by filtration and stored at $-24^{\circ}C$ in small aliquots. Every 100 ml of a growth medium received 0.2ml of this stock solution which contained $10^4$ U.penicillin and $10^4$µg streptomycin, respectively.

Gentamycin[2] was used from a readily made solution of ten mg per ml. To a 100ml of growth medium 0.75ml of gentamycin, the average amount recommended by the supplier was used. Gentamycin was either substituted for PS or it was used in addition to them, appropriately.

Adjustment of pH.

Growth media were buffered in three ways.

(a) A solution of 4.4 per cent w/v of sodium bicarbonate in DDW was prepared. This was autoclaved at 15 lb. per sq.inch for 15 minutes and stored at $+4^{\circ}C$. This solution was used to achieve the required pH in the media. Where reduction of pH was required 1/10 N sterile hydrochloric acid was used.

(b) Sodium bicarbonate plus carbon dioxide was used when cultures were enclosed in a sealed polythene box provided with two holes in the lid, the medium having been adjusted to the required pH with

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(1) Glaxo Laboratories Ltd., Greenford, England.

(2) Flow Laboratories Ltd.

sodium bicarbonate solution (a). Carbon dioxide gas to the extent of five per cent of the volume of the box was blown into the box and the holes were then sealed.

(c) Sodium bicarbonate was used to the required amount and Hepes buffer[1] (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid) was added. (Good, Winget, Winter, Connolly, Izawa and Singh 1966). The Hepes buffer was added at the rate of ten mM or 20 mM to medium containing Hank's or Earle's salts respectively, using a stock solution containing 1/10 M Hepes in 100 ml DDW (23.83g per cent solution w/v). This was sterilised by filtration and stored at +4°C. Final adjustment of the medium was achieved using 1/5 N sterile solution of sodium hydroxide. This was done on the recommendation of the suppliers as high concentrations of sodium bicarbonate exhaust the buffering capacity of Hepes buffer.

Sterility of media.

All media were tested for sterility by incubation of five ml aliquots.

5. Other reagents.

(a) Hank's and Earle's balanced salt solutions (BSS).[1]

(b) Phosphate buffered saline (PBS)[2], was made up using Dulbecco 'A' tablets according to the Oxoid Manual instructions.

(c) Alsever's solution was made up according to Dacie and Lewis (1970). This contained 2.05g glucose, 0.8g dehydrated trisodium citrate, 0.42g sodium chloride and 0.05g citric acid in 100ml DDW.

(d) Ammonium chloride solution was made up as recommended by

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(1) Flow Laboratories Ltd.

(2) Oxoid Ltd., London.

Odajima and Sonoda (1971). This contained 4.15g ammonium chloride in 500ml DDW.

(e) Sodium chloride solution was made up as a 5.4g per cent w/v in DDW, and also as a 0.8g per cent w/v solution in PBS as described by Carlson and Kaneko (1973).

(f) Sodium citrate was made up as a 3.8 per cent w/v solution in DDW according to Odajima and Sonoda (1971). Another solution was also made up by dissolving 0.95g sodium citrate in 100 ml DDW.

(g) A solution of diamino-ethane-tetra-acetic acid (EDTA or versene)[1] was made up at the rate of 200 mg per litre in PBS as described by Paul (1975). The solution was sterilised by filtration.

(h) Trypsin solution was made up as a concentration of 0.25 per cent w/v trysin (1/250 Difco) in Ca, Mg-free PBS solution according to Paul (1975). This solution was sterilised by filtration and stored at -24°C.

(i) Cryoprotectants used, were Analar glycerol and Analar dimethyl sulphoxide (DMSO)[2].

(j) Phytohaemagglutinin (PHA)[3], as the mitogen was reconstituted by the addition of five ml sterile DDW to the freeze-dried content of a bottle. This was used within one month as recommended by the supplier.

(k) Colcemid, pure substance, (Demicolcine)[4] was made up as a 0.02 per cent w/v solution in DDW. This was sterilised by filtration and kept at + 4°C.

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(1) Sodium versenate supplied by Difco Laboratories Ltd.

(2) BDH Chemicals Ltd.

(3) Wellcome Reagents Ltd.

(4) Ciba Laboratories Ltd., Horsham, Sussex.

(l) A hypotonic solution of potassium chloride was made up by dissolving 0.56g KCl in 100 ml DDW, and it was sterilised by filtration.

(m) The fixative used, consisted of three parts of methyl alcohol and one part of glacial acetic acid mixed immediately prior to use.

(n) Sodium metrizoate/Ficoll solution (Lymphoprep)[1].

6. Sterilisation of tissue culture materials.

Disposable utensils, such as tissue culture flasks, bottles, petri dishes and syringes were used as obtained sterile from manufacturers.

All glassware used in tissue culture was soaked in a one per cent solution of Famosan[2], as recommended by the Ministry of Agriculture to prevent any possible contamination with Foot and Mouth or Swine Vesicular Diseases. The glassware and rubber stoppers were soaked in this solution overnight or longer but, metal caps and other metal instruments were immersed for one hour only.

The glassware was washed in one to two per cent dilution of Decon 75[3], immersed in tap water for 30 to 60 minutes, and rinsed three times in deionised distilled water (DDW).

Pipettes were immersed in Famosan and Decon and then washed in a syphon type pipette-washer. These were then rinsed three times in distilled water.

Slides and coverslips were placed in special racks and

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(1) Pharmacia Fine Chemicals, Uppsala, Sweden.

(2) Wellcome Reagents, Wellcome Foundation Ltd., Berkhamsted, England.

(3) Decon Laboratories Ltd., Ellen Street, Portslane, Brighton, England.

immersed in a one per cent dilution of Decon 75, overnight, or they were boiled for one hour in this detergent. They were then placed in running tap water for one hour and after three rinses in DDW they were dried in an oven.

The washed glassware and metal tools were sterilised at 160°C for two hours in a dry heat oven.

The rubber stoppers, rubber tubing and glassware with gauze plugs were autoclaved at 15 lb. per sq.inch for 15 minutes. The chemicals and salt solutions which are not heat labile were autoclaved at 15 lb. per sq. inch for 15 minutes. The other materials like amino-acids, serum and reagents which are heat labile were sterilised by filtration using sintered glass filters[(1)] or Millipore filters[(2)].

7. Uninfected foreign cells.

(a) Baby hamster kidney cells, BHK-21 (C13)[(3)].

(b) African green monkey cells (Vero)[(3)].

(c) Horse leucocytes obtained from defibrinated horse blood.[(4)]

(d) Sheep leucocytes and

(e) rabbit leucocytes, both were prepared from stock animals at the C.T.V.M.

In addition, washed sheep and bovine red blood cells were

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(1) Corning Glass Works, Corning, N.Y., U.S.A.

(2) Millipore, U.K. Ltd., Wembley, Middlesex.

(3) Gibco:Bio-cult Ltd., Sandyford Industrial Estate, Paisley, Scotland.

(4) Wellcome Reagents Ltd.

prepared in Alsever's solution or PBS; Ca,Mg-free solution.

8. Preparation of normal uninfected lymphoid cells from bovine lymph node and spleen.

Prescapular lymph nodes from slaughtered calves were placed in ice-cold medium 199 at a pH of 7.2. They were stripped aseptically and washed several times in Hank's salt solution containing antibiotics. The lymphocytes were then removed by one of three methods:-

(a) The injection and re-aspiration of medium 199.

(b) The chopping and subsequent scraping of the cut surfaces into medium which was then filtered through a 0.24 $mm^2$ stainless steel wire mesh and a multilayer muslin sieve.

(c) The immersion of the whole node in medium 199 and mincing with sharp scissors followed by filtration through several layers of muslin.

Method (c) produced the richest suspension of cells of which 95 per cent were viable.

Spleen cells were taken from calves and adult cattle after slaughter. Lymphocytes were obtained in suspension in medium 199 in the same way as for those derived from lymph nodes. The lymphocytes were separated from the red cells by repeated washing and centrifugation.

9. Preparation of lymph node extract (LNE) from normal cattle.

Prescapular lymph nodes from healthy cattle were used. Two nodes were minced thoroughly in ten ml of medium 199 as in (c) above. The pulp was filtered through a 0.24 $mm^2$ stainless wire mesh. The fluid was collected and the remaining pulp was minced

again. This was thoroughly ground in adequate medium in a Tenbruk grinder. Finally the whole mixture was centrifuged at 2000 G for 30 minutes. The supernatant fluid was collected and filtered through a 0.22 µm millipore filter. The LNE was dispensed in small aliquots and stored at -24°C.

10. Preparation of defibrinated and non-coagulated blood.

(i) Jugular blood was drawn into an Erlenmayer flask, defibrination being brought about by agitation with glass beads. The blood was then filtered through a sterile gauze pad and antibiotics including gentamycin were added.

(ii) Non-coagulated blood was prepared either by mixing 20 per cent v/v of a 3.8 per cent w/v sodium citrate solution, with blood withdrawn from the jugular vein, or by means of heparinised Vacutainers.(1) Every Vacutainer contained 143 USP Units of sodium heparin for seven ml blood. Antibiotics were added to the blood before use.

11. Preparation of schizont-infected cells from infected tissues, biopsy and autopsy materials.

(a) Lymph node biopsy was carried out as described by Sergent, Donatien, Parrot, Lestoquard, Plantureax and Rougebief (1924a and 1924b), who followed the technique of East African workers. A needle, 16 to 20 G and five to ten cm long was used. The needle was attached to a syringe and some 0.5ml medium was injected into the lymph node after which, fluid was aspirated.

(b) Liver biopsy was also performed according to Sergent et al

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(1) Vacutaine system B-D, France.

(1924a and 1924b) who followed the technique of East African workers. Entry was made at the eleventh intercostal space on the right-hand side of the animal, about four cm below the horizontal line which crosses the external angle of the ilium. The needle used, was a ten cm long, 16 G fitted with a stilette. The animal was completely restrained. Without any pre-incision of the skin, the needle was inserted through the skin and muscle and pushed downward and forward along the sternum until it penetrated liver tissue. Liver tissue was withdrawn in the needle and was expelled into medium in a test tube.

Infected blood, lymph node and liver tissues from sick cattle were used for isolation of the parasite (Chapter 4) and for preparation of stained smears.

(c) Material was also obtained from lymph node, liver and spleen of animals immediately after slaughter.

The procedure for separation of schizont-infected cells from these infected tissues for isolation purposes was as described for normal cells.

12. Separation of leucocytes from bovine peripheral blood.

Similar procedures were used whether the blood was from normal or infected animals.

(a) By cell adhesion.

The method used was as described by Tokuda et al (1962) with some modification. Antibiotics were added to the whole blood, either defibrinated or non-coagulated, after which it was incubated at 37°C for three days, the tubes being sloped at an angle of approximately 40°C. The red cells sedimented thus facilitating the attachment of the white cells to the surfaces of the tubes.

Free cells were then washed off and the attached leucocytes were set up in culture vessels.

(b) By lysis of red blood cells with DDW or ammonium chloride solution.

The method, using DDW, was as described by Odajima and Sonoda (1970) and that using ammonium chloride was as described by the same authors (1971).

(c) Separation of pure lymphocytes from bovine peripheral blood.

A number of gradient media were tested and sodium metrizoate/ Ficoll solution, (Lymphoprep)[1] was chosen and used for this purpose. Two ml of defibrinated or non-coagulated blood was mixed with an equal volume of PBS solution. This was layered gently on three ml of Lymphoprep in a ten ml centrifuge tube and centrifuged at 400 G for 30 minutes at 18°C. An interface layer, consisting of pure lymphocytes appeared which was sucked out, Figs. 3.1 and 3.2. The lymphocytes were washed with PBS and spun down at 160 G for ten minutes, twice. The sedimented cells were resuspended in fresh medium and used.

A similar procedure was used for separating viable schizont-infected from non-viable cells, whether these were from thawed stabilates or from already established culture, Figs. 3.3 and 3.4.

13. Viability test of normal and schizont-infected lymphocytes.

The dye exclusion test was adopted (Weir 1974; Ling and Kay 1975). Eosin Y, nigrosin and trypan blue were tested and the latter was chosen as the best. Three concentrations, 0.8, 0.4 and 0.2 per cent w/v of trypan blue were made up in PBS with a pH of

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(1) Pharmacia Fine Chemicals.

7.2, (Paul 1975) as stock solutions. It was found that for schizont-infected cells a final concentration of 0.05 per cent was required and for normal lymphocytes a final concentration of 0.2 per cent trypan blue was required.

These were used for one minute and five minutes, respectively. The concentration of the stain and the percentage of CS in the medium were adjusted to the same level in all tests.

For schizont-infected cells two parts of cell suspension containing ten per cent CS, one part of growth medium containing 20 per cent CS and one part of a 0.2 per cent of stain were mixed. For normal lymphocytes similar proportions were used but the stain was at the strength of 0.8 per cent. Non-viable cells stained dark and viable cells excluded the stain and remained refractile, Fig.3.5. Counting was carried out in an improved Neubauer haemocytometer.

14. Preparation of rabbit anti-bovine lymphocyte antiserum (RABLS).

The RABLS was prepared according to Weir (1974) as follows:- Normal lymphoid cells were prepared from prescapular lymph nodes of a calf as described above. Viable cells at the level of $1.0 \times 10^9$ cells per ml of medium were resuspended in one ml of growth medium. This was inoculated intravenously (i/v) into a rabbit. The rabbit was re-inoculated i/v after two weeks with the same number of lymph node cells from a normal calf. After one further week the rabbit was bled and the serum was separated. This was then distributed in small aliquots in Bijou bottles. The serum in some of the bottles was inactivated by heating in a 56°C water-bath for 30 minutes, and some other aliquots were kept unheated. All of

them were stored at -24°C until used. Normal serum was prepared by bleeding a healthy rabbit as a control and kept at -24°C.

15. Trypsinisation of the cultures.

The culture medium was removed and the surfaces of the culture vessels were washed twice with calcium and magnesium-free PBS. Adequate amounts of versene or trypsin solutions were added to the culture vessels to cover the cells attached to the surface. They were then incubated at 37°C for five to ten minutes. Each culture vessel was examined, using an inverted microscope, at intervals. The cells were detached by shaking and two to five ml calf serum, depending on the volume of the vessel, were added to stop the action of the versene or trypsin. Growth medium was added to the cell suspension which was centrifuged at 160 G for five minutes. The cells were then resuspended in fresh medium at the desired concentration.

16. Preparation of chromosomes from schizont-infected cells and normal cells from cattle.

Several methods for the demonstration and examination of bovine chromosomes have been described (Axelrad and McCulloch 1958; Rothfels and Siminovitch 1958; Moorhead, Nowell, Mellman, Battips and Hungerford 1960; Kosenow and Pfeiffer 1961; Nichols, Levan and Lawrence 1962; Sasaki and Makino 1962; Biggers and McFeely 1963; Ulbrich, Weinhold and Pfeiffer 1963). After testing several methods and on the advice of R. Buckland of the Western General Hospital, Edinburgh, the following technique was adopted.

(i) To five ml of schizont-infected lymphoid cells, five per cent v/v of colcemid solution was added and mixed thoroughly. The

culture was re-incubated at 37°C for three hours, in order to arrest mitosis. The cell culture was then spun down at approximately 1000 G for five minutes and the supernatant fluid was gently decanted. To the cell pellet eight ml of hypotonic solution, either potassium chloride or sodium citrate were added. The cells were pipetted and brought to suspension and left at room temperature for eight minutes. The culture was centrifuged as before and the supernatant fluid was discarded. To the deposited cells two ml of fixative, (three parts of methanol and one part of glacial acetic acid freshly prepared) were added dropwise, the tube being shaken by an electric Whirlimixer.[1] The cells were centrifuged and the supernatant fluid was discarded. This process was repeated twice. After the third centrifugation, the supernate was decanted and 0.5 ml of fresh fixative was added to the deposit which was brought to suspension. From this cell suspension drop or linear smears were prepared on clean slides. The smears were air dried and stained with Giemsa for examination under a x50 oil immersion objective with a x15 eyepiece.

(ii) Normal cells from cattle used for chromosome preparation were (a) blood leucocytes and (b) lymph node and spleen cells.

(a) In several preliminary trials it was shown that there was no difference in result whether leucocytes separated from blood, or leucocytes in whole blood were used. For convenience, therefore, whole blood was used. Defibrinated or non-coagulated blood from normal male and female cattle were used at the rate of 0.4 ml whole blood in ten ml growth medium. The medium used was Eagle's

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(1) Fisons Scientific Apparatus, Loughborough, Leicestershire, England.

MEM supplemented with CS and LY, as previously described. Each of the blood cell cultures which was set up in a plastic Universal centrifuge tube, received 1.6 per cent v/v PHA solution as stimulant for cell mitosis. The cultures were incubated at 37°C. After three days of cultivation 0.5 ml of the colcemid solution was added to each of the ten ml cultures. These were processed as in (i) above.

(b) Suspension cells separated from lymph nodes and spleen of normal cattle were adjusted separately to $1.0 \times 10^6$ cells per ml of medium which was as in (a) above. PHA solution was added to the level of 1.6 per cent v/v and the procedure followed was as in (i) and (ii,a) above.

RESULTS

(i) Schizont-infected cells, S.3 and S.15 were confirmed to have been derived from male cattle. The schizont particles were found always associated with the chromosomes, Fig. 3.6 and Fig. 3.7.

(ii) It was shown that whole blood can be utilised easily for chromosome preparation from leucocytes, without the necessity for separating WBC from rbc. This method of chromosome preparation was a simple and reliable means of demonstrating cell mitosis and the sex of the cell donor animal, Fig. 3.8 and Fig. 3.9.

17. Measurement of glucose uptake and lactate production in the cell culture.

For the measurements of glucose and lactate, the following steps were carried out:-

(a) Sampling for glucose estimation.

(b) Sampling for lactate estimation.

(c) Calibration of optical density for glucose determination.

(d) Calibration of optical density for lactate determination.

(a) Sampling for glucose estimation.

The samples of the cell cultures, at appropriate intervals, were centrifuged at approximately 1000 G for seven minutes. The supernatant fluids were carefully decanted into Bijou bottles and cell deposits were used for preparing stained smears.

The method used for deproteinisation of samples for glucose and lactate estimation was based on the principle of Henry (1964), using the GOD-POD colorimetric method. An amount of 0.5 ml of each fluid was pipetted into a 15 ml centrifuge tube containing five ml of 0.33 N perchloric acid. This was done to deproteinise the supernatant fluids. The contents were mixed thoroughly and centrifuged at approximately 2000 G for ten minutes. The deproteinised supernates were gently poured into Bijou bottles and stored at $-24^{\circ}C$, until all estimations could be made.

(b) Sampling for lactate estimation.

One ml of the supernatant fluids, prepared previously, was mixed with two ml ice-cold 0.6 N perchloric acid in a 15 ml centrifuge tube. After pipetting and mixing thoroughly, the samples were centrifuged at 2000 G for ten minutes. The deproteinised supernatant solutions were carefully decanted into Bijou bottles and stored at $-24^{\circ}C$ until all samples could be estimated at one time.

(c) Calibration of optical density (O.D.) for glucose determination.

The measurement of glucose from prepared samples (Chapter 8) was made using the Sclavo Glu-cinet test kit.[1] The estimation

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(1) Sclavo Glu-cinet test kit supplied by A.J. Seward, Bury St. Edmonds Suffolk.

was based on the GOD-POD colorimetric method for glucose in blood. The whole procedure of sampling and estimation followed the test kit instructions, Trinder (1969). The standard solution of the test kit which contains 100 mg D-glucose per 100 ml benzoic acid was calibrated as follows:- One g Analar, (AR) glucose was weighed and dissolved in 100 ml DDW. This gave a concentration of 10.0 mg glucose per ml. This was referred to as the stock standard, which was left at room temperature for three hours. Five sub-standard dilutions were made by dispensing 1.0, 2.0, 3.0, 4.0 and 5.0 ml of the stock standard into five flasks and making the volume of each one up to 100, using DDW. These sub-standard solutions contain: 10.0, 20.0, 30.0, 40.0 and 50.0 mg glucose per 100 DDW, respectively. From each of these glucose dilutions four samples were made in addition to a 1:10 dilution of D-glucose of the test kit standard. The samples were read at 510 nm against the blank specimen using a Unicam SP 1800 ultraspectrophotometer.[1]

RESULTS

The mean optical densities for mg of glucose per 100 ml of various dilutions are shown in Table 3.1 and Fig. 3.10.

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(1) PYE, Unicam, York Street, Cambridge.

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Table 3.1 Calibration of optical density for glucose determination.

| Sample | mg glucose/100 ml | Mean O.D. |
|---|---|---|
| | 0.0 | 0.000 |
| A.R. Glucose | 10.0 | 0.221 |
| " " | 20.0 | 0.451 |
| " " | 30.0 | 0.678 |
| " " | 40.0 | 0.851 |
| " " | 50.0 | 1.145 |
| D-glucose test-kit standard | 10.0 | 0.224 |

The values presented a straight line, within the limits of mean deviation.

(d) Calibration of optical density (O.D.) for lactate determination.

The preparation of samples and lactate estimation were made using the Biochemica Test Combination.(1) For the calibration control, the Lactate Calibration Solution (L.C.S.), Biochemica Test Combination, was applied. According to the test kit, 0.2 ml of L.C.S., when diluted 1:2000 in 0.6 N perchloric acid contains 4.5 mg lactate per 100 ml. As the control test, four dilutions of 1:500, 1:1000, 1:2000 and 1:4000 which contained 18, 9, 4.5 and 2.25 mg lactate per 100 ml, respectively, were prepared. From each of these dilutions and a test kit blank containing 0.6 N perchloric acid, 0.2 ml were used and the blank samples were made according to the test kit instructions. Each of these samples was read against air at a wavelength 340 nm.

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(1) Boehringer Corporation, London.

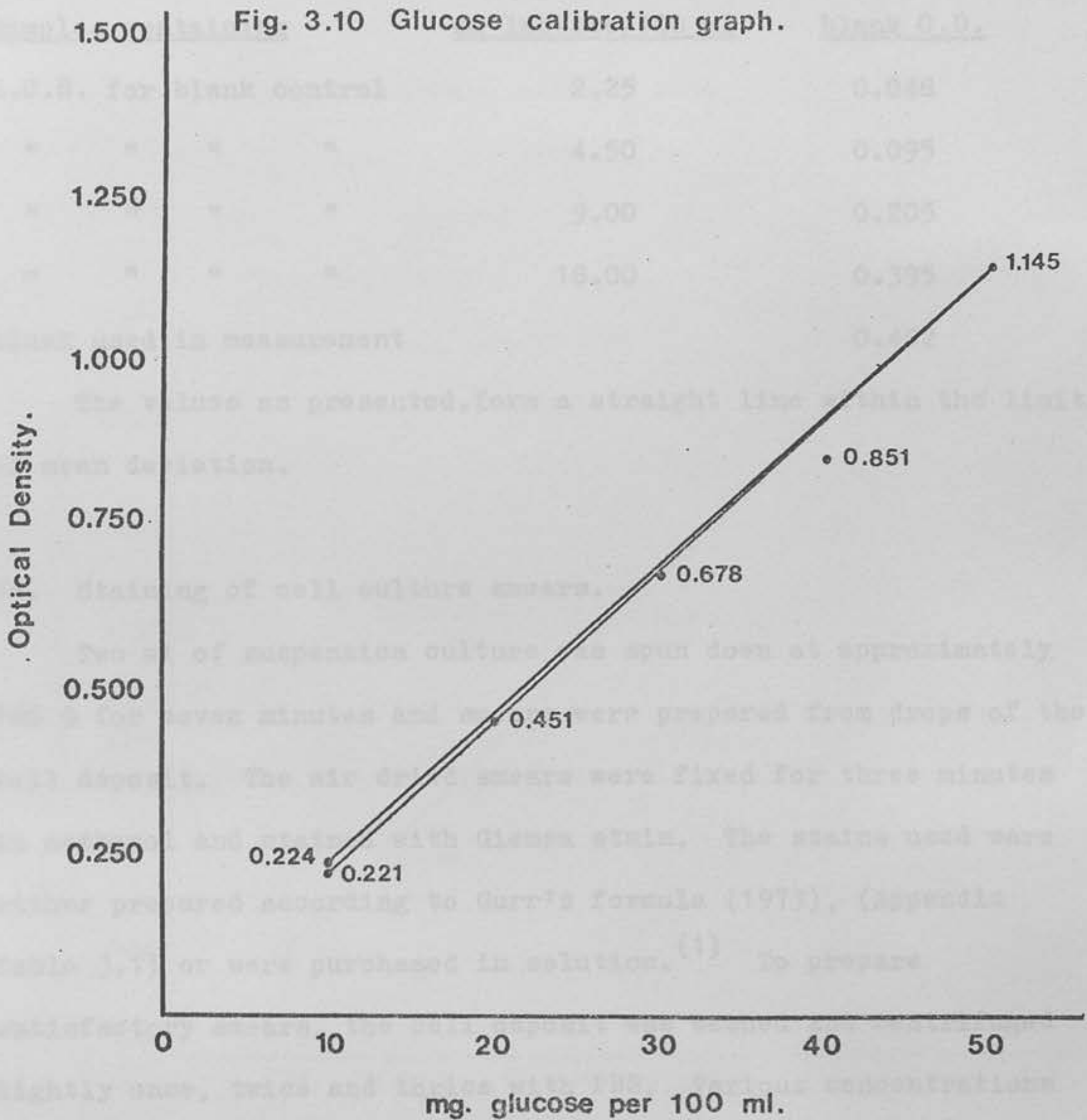


Fig. 3.10 Glucose calibration graph.

RESULTS

The optical densities and the doubling ranges of mg lactate per 100 ml are shown in table 3.2 and Fig. 3.11.

Table 3.2 Calibration of optical density (O.D.) for lactate determination.

| Samples containing | mg lactate/100 ml | blank O.D. |
|---|---|---|
| L.C.S. for blank control | 2.25 | 0.048 |
| " " " " | 4.50 | 0.095 |
| " " " " | 9.00 | 0.205 |
| " " " " | 18.00 | 0.395 |
| blank used in measurement | | 0.402 |

The values as presented,form a straight line within the limits of mean deviation.

18. Staining of cell culture smears.

Two ml of suspension culture was spun down at approximately 160 G for seven minutes and smears were prepared from drops of the cell deposit. The air dried smears were fixed for three minutes in methanol and stained with Giemsa stain. The stains used were either prepared according to Gurr's formula (1973), (Appendix Table 3.1) or were purchased in solution.[1] To prepare satisfactory smears, the cell deposit was washed and centrifuged lightly once, twice and thrice with PBS. Various concentrations of stain and times of exposure were used. (i) Five per cent Giemsa for 30 minutes, (ii) five per cent stain for 45 minutes, (iii) 7.5 per cent stain for 30 minutes and (iv) ten per cent

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(1) Gurr's Improved R66 supplied by: Gurr Products, High Wycombe, Bucks., England.

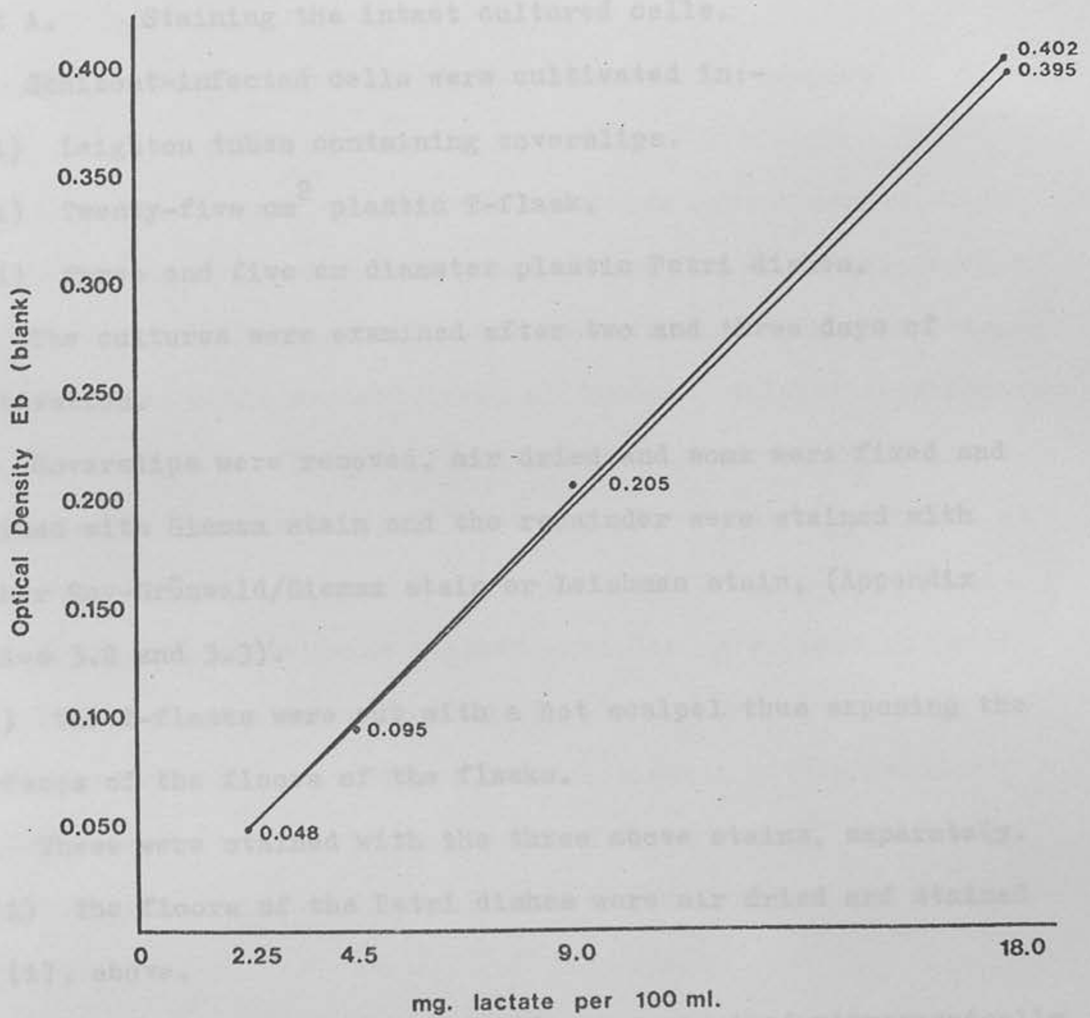



Fig. 3.11 Lactate calibration graph.

stain for 20 minutes. None of the smears stained by these methods were satisfactory as the schizonts within the cell cytoplasm were not clearly distinguishable. Similarly stained smears from blood and lymph nodes showed the morphology of the schizonts clearly.

The following tests were carried out in order to determine a suitable method of demonstrating cultured cells with as little disturbance as possible.

Test A. Staining the intact cultured cells.

Schizont-infected cells were cultivated in:-

(i) Leighton tubes containing coverslips.

(ii) Twenty-five $cm^2$ plastic T-flask.

(iii) Three and five cm diameter plastic Petri dishes.

The cultures were examined after two and three days of cultivation.

(i) Coverslips were removed, air dried and some were fixed and stained with Giemsa stain and the remainder were stained with either May-Grünwald/Giemsa stain or Leishman stain, (Appendix Tables 3.2 and 3.3).

(ii) The T-flasks were cut with a hot scalpel thus exposing the surfaces of the floors of the flasks.

These were stained with the three above stains, separately.

(iii) The floors of the Petri dishes were air dried and stained as (i), above.

The stained films, so produced, were examined microscopically.

As a result, schizonts were discernible in some 50 per cent of the host cells. The cells were found spread irregularly on the surfaces, so that most of the overcrowded cells were heavily stained and their structures were not well distinguishable. The May-Grünwald/Giemsa and Leishman stains showed no superiority over

Giemsa stain. Thus, in subsequent work, Giemsa stain was used as it was simpler and more convenient.

This method of making stained preparations of schizont-infected cells was not very satisfactory for routine examination of cultures.

Tests were, therefore, carried out with suspensions of cells, smeared in various ways on microscope slides.

Test B. Standardisation of the technique to prepare smears from suspension culture.

Many trials were carried out to decide upon a consistently satisfactory method of smearing and staining. It was clear that unless the smear preparations were consistent, in that an even spread of the cells was obtained, a standard staining technique was not possible. Thus, the cell culture was centrifuged and a suspension of the cell deposit was used for smear preparation as follows:-

(i) Smears of drops using a glass spreader or a glass rod bent at right angles at its extremity.

(ii) Smears made by placing a drop on a slide with a Pasteur pipette and spreading with a glass rod to an area of 1 x 2 $cm^2$.

(iii) A small droplet from a Pasteur pipette spread by various spreaders.

(iv) A large intact drop from a Pasteur pipette.

(v) Droplet from Pasteur pipette tapered to a fine capillary, the excess being reclaimed with the same pipette.

(vi) Sedimentary material collected in a fine capillary tube and then allowed to draw itself out by contact as the tube was drawn along the slide.

All of the smears were air dried and stained with 5.0 per cent Giemsa stain for 30 minutes. As a result, the method (vi) for preparation of culture smears, proved to be the best, giving fast drying and consistently uniform uncrowded smears. Schizont-infected cells could be clearly distinguished in most preparations.

Test C. Standardisation of the staining methods of the cell culture smears.

For routine purposes, cell suspension smears were prepared as in (vi) above. These were fixed and stained with Giemsa stain in thirty combinations of stain concentrations and time of exposure from 0.25 per cent Giemsa for 48 hours to 20.0 per cent Giemsa for one minute. After staining, the slides were washed in Giemsa buffer (pH 7.0) three times, dried and examined. Optimum results were given by using 0.5 per cent stain overnight (18 to 24 hours), 5.0 per cent stain for 30 minutes and 10.0 per cent stain for ten minutes. There was no observable difference in the quality of the stained cells by these three methods. It was, however, found that the proportion of damaged or non-viable cells was higher in these linear smears than in the cell suspension from which they were derived. Consideration was, therefore, given to two possible reasons for this. The first was the possible damage due to centrifugation. Observation on suspensions centrifuged at different rates (120 G to 100,000 G) indicated clearly that this factor had little effect on the proportion of damaged or non-viable cells. The second was the calibre of the capillary tube used for making the smears. Tests were made using capillaries of calibre 25 μm to 150 μm. The smears with the optimum numbers of intact cells were those made using capillaries of between 50 μm

and 90 µm. Capillaries of this range of calibre were, therefore, adopted for routine smear preparations. A simple apparatus was designed so that the suspension could be blown gently from the tube as the opening of this was being drawn along the slide. This is illustrated in Fig. 3.12. When smears made by this method were stained, the results were very good, the cells and their included schizonts being clearly defined. This made possible the measurement of the proportion of schizont-infected cells in any culture suspension.

When bovine red blood cells are added to the cell suspension, schizont-infected cells stain more clearly, (D. Danskin personal communication). It was confirmed repeatedly that by the addition of a droplet of 1:100 bovine washed rbc to the sedimented suspension cells, these stained well in both spread smears and linear smears. It appeared that the presence of rbc, improves staining of lymphocytic cells or simply causes them to spread well with consequently better staining.

Attempts were made to see if other additives would improve the staining quality of the cells without the use of other cells. Accordingly, smears were made using five per cent and ten per cent glucose, dextran and bovine plasma albumin as additives. These did not show improvements in the stained smears comparable with that produced by the addition of washed red cells.

However, the use of a fine capillary aspirator for preparing linear smears was found helpful in several ways including, obtaining even-spread cells which could be stained well, and in scanning the cells and locating them. Using this method for preparation of smears and staining these with any of the three combinations of stain concentration and exposure time (c) above,

it was often found that 100 per cent of the cells included schizonts (Figs. 3.13 and 3.14).

Figs. 3.1 and 3.2

Separation of blood lymphocytes by gradient centrifugation using Lymphoprep.

3.1 (above). Before centrifugation.

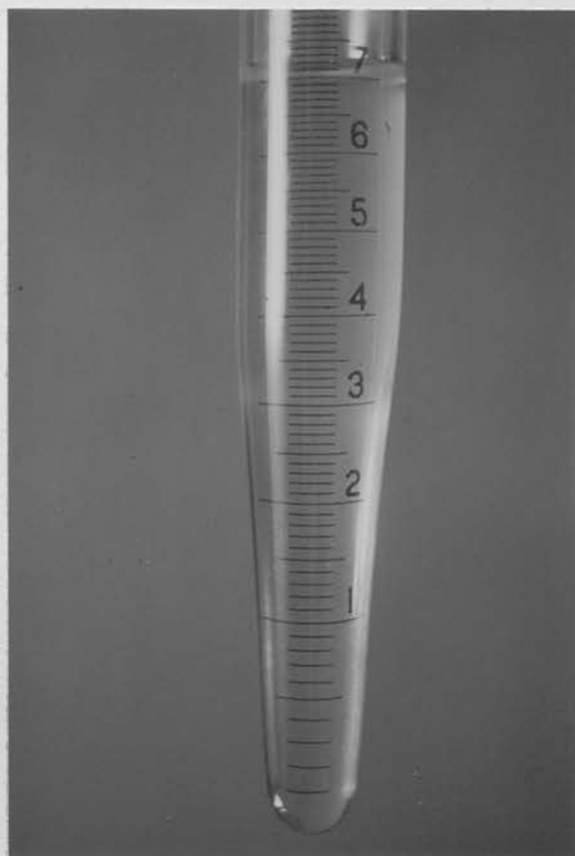
3.2 (below). After centrifugation. The lymphocyte layer at the interface is indicated by the arrow.

Figs. 3.3 and 3.4

Separation of viable schizont-infected cells from the non-viable cells by gradient centrifugation using Lymphoprep.

3.3 (above). Before centrifugation.

3.4 (below). After centrifugation. The layer of the viable cells at the interface and the non-viable cells at the bottom of the tube are arrowed.


7
6
5
4
3
2
1

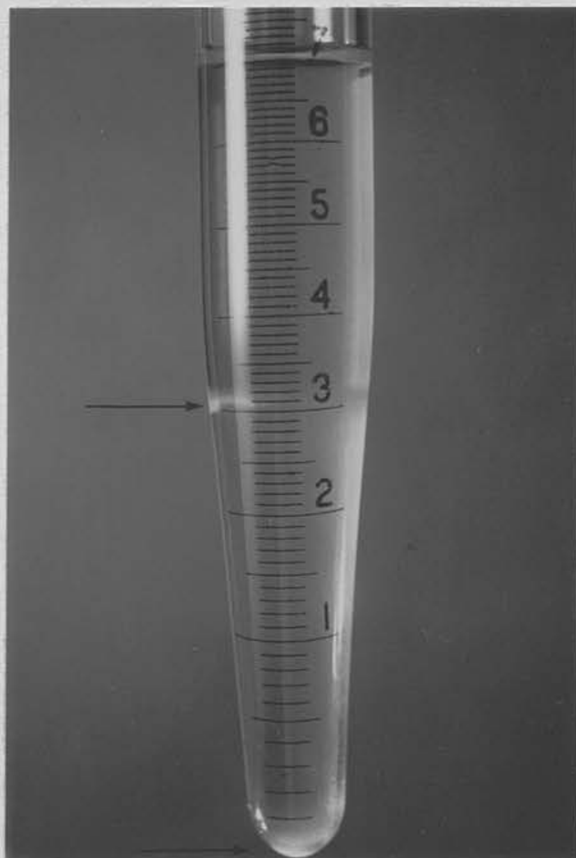
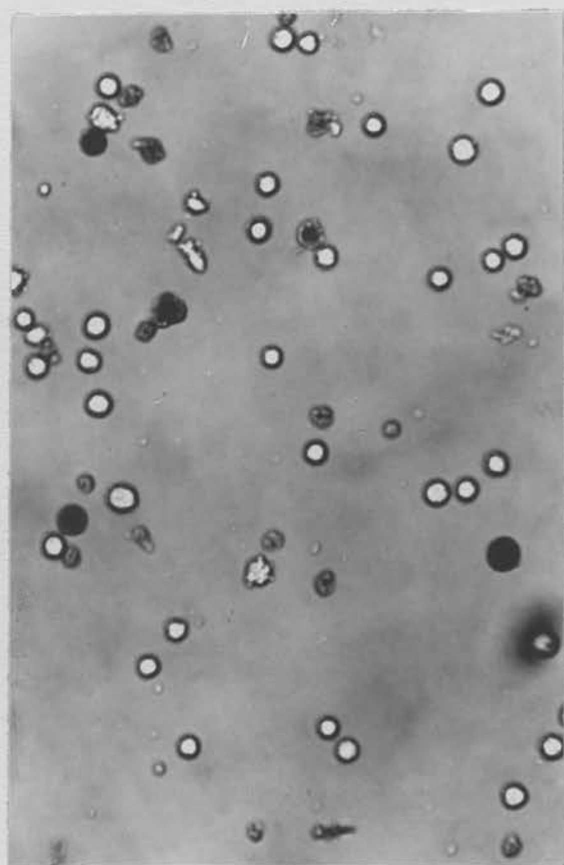

7
6
5
4
3
2
1

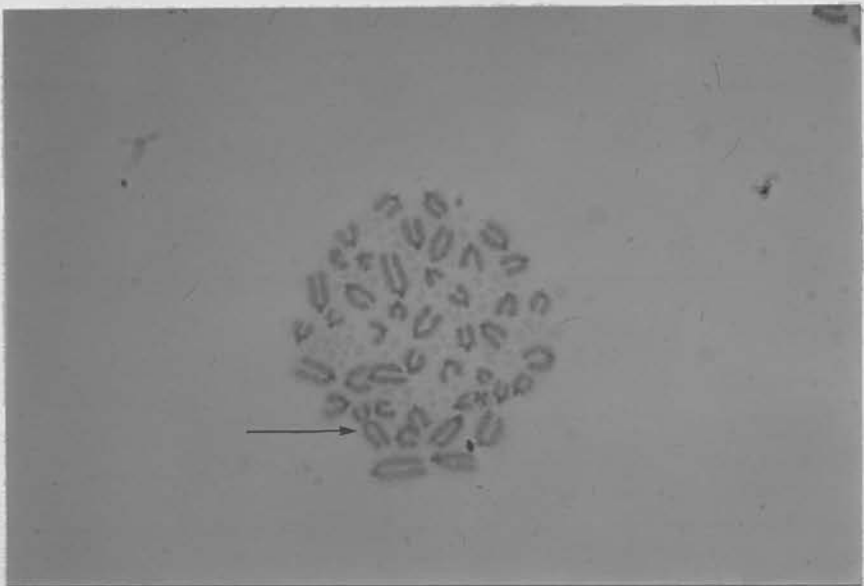
Fig. 3.5

Viability test using trypan blue. Viable schizont-infected cells exclude the stain and remain refractile and the non-viable cells stain darkly.



Figs. 3.6 and 3.7

Chromosomes in *T. annulata*-infected cells from male cattle (S.3 above and S.15 below). One metacentric or X chromosome is present and shown by the arrow. The schizonts are associated with the chromosomes.



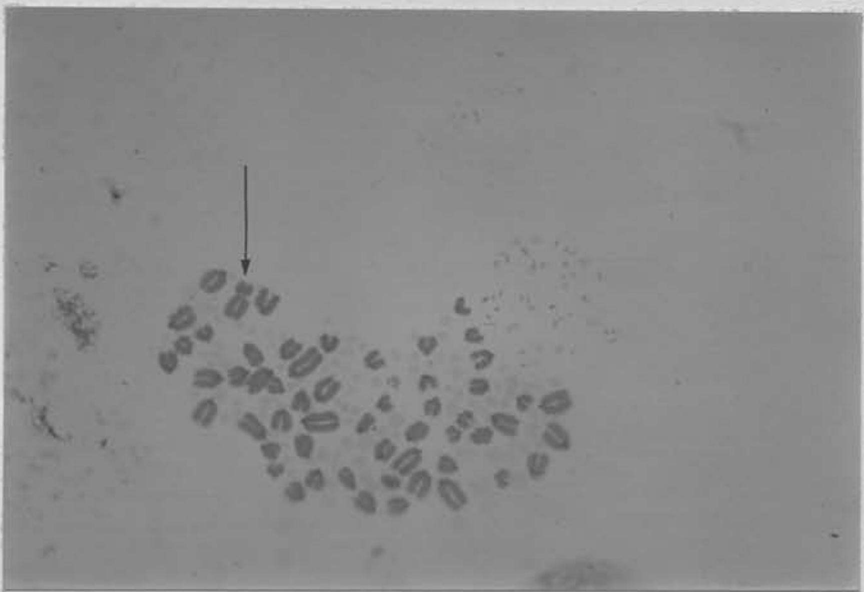


Fig. 3.8

Chromosomes in leucocytes of a normal male calf. One X chromosome is indicated by the arrow.

Fig. 3.9

Chromosomes in leucocytes of a normal cow. Two X chromosomes are shown by the arrows.

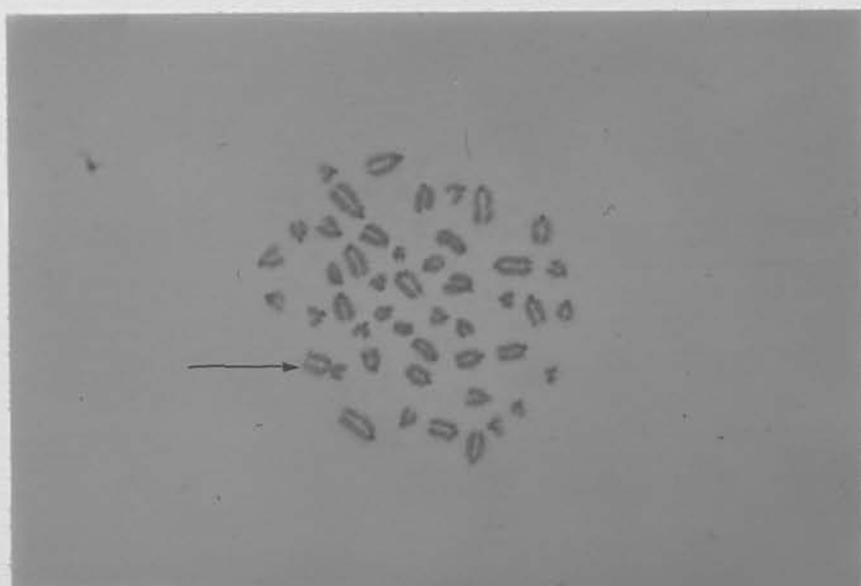
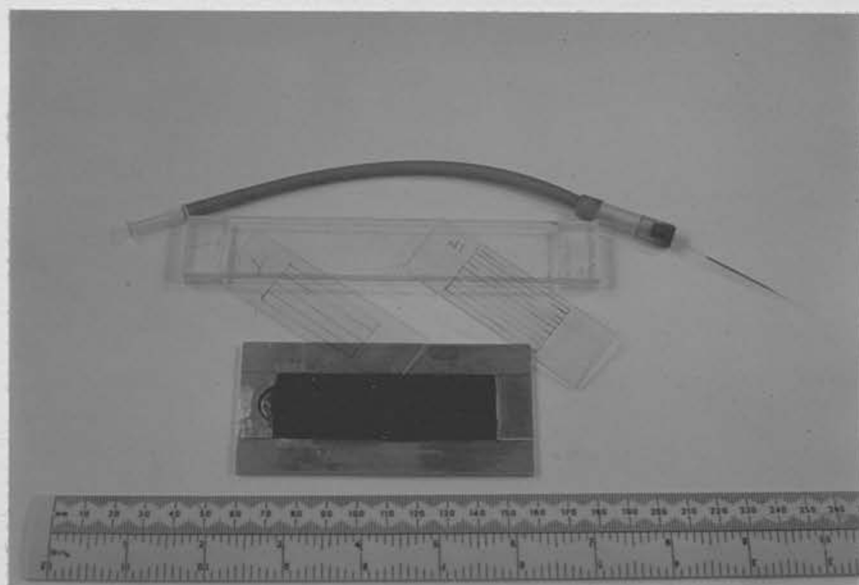


Fig. 3.12

A capillary tube aspirator adopted for preparation of linear smears.

Fig. 3.13

A linear smear of suspension cell culture stained with Giemsa.



# CHAPTER 4

## ISOLATION OF *THEILERIA ANNULATA* STRAINS

A. From biopsy materials.

I. Isolation from the blood.

The following experiments were carried out to determine whether or not the infective agent can be isolated from infective blood in suspension culture.

Experiment 4.1

Isolation of strain 3 (S.3) using lysed blood.

A twelve month old, male, holstein calf No. 2539 infected with *T. annulata*, (S.3) was used. Eighteen days after inoculation the temperature of the animal had risen to 40.6°C. Stained smears from the prescapular lymph node showed ten to 15 schizont-infected cells per microscopic field as well as free schizonts. In stained smears from peripheral blood, frequent intra-lymphocytic and free schizonts were seen. No erythrocytic forms were observed, as this strain does not produce piroplasms. At this stage the calf was bled and citrated blood was prepared as described in Chapter 3. The leucocytes were separated by lysing the red blood cells using ammonium chloride solution according to the method of Odajima and Sonoda (1971). These leucocytes were resuspended in fresh medium and adjusted to contain 0.5, 1.0 and 2.0 x $10^6$ cells per ml in duplicate cultures. They were then incubated separately, in tightly stoppered one ounce bottles at 37°C. The medium used was a modified Eagle's described by Hooshmand-Rad (1973) and supplemented with LY as explained in Chapter 3. The pH of the

medium was adjusted to 7.2 at 37°C using sodium bicarbonate buffer. The cultures were checked on an inverted microscope and stained samples of the schizont-infected cells were prepared and examined daily. All of these cultures were made in suspension and were subcultured twice weekly. On some occasions, as appropriate, fresh medium was added to the cultures or sub-culturing was postponed. The expansion of the cultures was carried out using $5.0 \times 10^5$ cells per ml in early passages when two and four ounce medical flats were used. Later still, cultures were transferred to Roux bottles.

RESULTS

After 24 hours, considerable numbers of infected cells were found dead or ruptured. In the following two to three days a slow increase in growth rate was observed. This was maintained until the cultures were passaged after six days. The cell increase varied slightly in different cultures, those seeded initially with $1.0 \times 10^6$ and $2.0 \times 10^6$ cells per ml established in 16 to 20 days but, the cultures initiated with $0.5 \times 10^6$ cells per ml did not establish despite maintenance for a period of four weeks. Infected cells in four out of six cultures established themselves. The details are shown in Table 4.1. (p 71)

Experiment 4.2

Isolation of strain 3 (S.3) using whole blood.

In this experiment attempts were made to isolate the schizont-infected cells from whole blood without any process of separation. The method was adopted with some modification from Tokuda et al (1962), as described in Chapter 3. Blood was taken

from calf No. 2539, on day 20 after inoculation, when it showed a temperature of 41.0°C. Stained smears from the prescapular node contained ten to 15 intra-lymphocytic schizonts per microscopic field and also free schizonts. Stained smears of the peripheral blood showed very frequent intra-cellular and free schizonts than before. Defibrinated blood was prepared for culture by the addition of antibiotics at the rate of $10^4$ units penicillin and 10 mg streptomycin per 100 ml. Some of this blood was distributed into 12 x 100 mm test tubes half receiving 0.5 ml and half receiving one ml. They were then stoppered tightly and set up at angles of 40° and 30°, respectively. These were marked and kept stationary in the incubator at 37°C. Coverslips were included in a proportion of the tubes so that they could be examined at different stages of cultivation. The remainder of the blood was distributed as ten ml aliquots into 8 x 12 cm Carrel flasks. These were stoppered and set up in an inclined position (25°-30°) in the incubator. The Carrel flasks and those test tubes without coverslips were incubated, untouched, for 72 hours. From the test tubes containing coverslips, these were removed, washed, stained and examined sequentially at 24, 48 and 72 hours. At 72 hours the plasma and free cells from the remaining cultures were carefully removed by pasteur pipette without disturbing the cells which had adhered. The internal surfaces were then washed very gently two or three times with medium in order to remove the erythrocytes and non-attached cells. Fresh medium was then added to the test tubes and flasks to replace exactly, by volume and in position, the original amounts of blood. The test tubes were set up in the same position as previously. The flasks were, however, laid flat and all were returned to the

incubator for four more days. Thereafter, the cultures were passaged and expanded. The cells which had adhered were brought into suspension by pipetting and agitation. Then, adjusting the number of cells to $2.0 \times 10^5$ per ml of culture, regular subculturing was carried out twice weekly.

RESULTS

Examination of the coverslips showed that after one day there were only a few schizont-infected cells attached. These cells were considerably increased in number after two and three days. During the second passage growth of the schizont-infected cells was observed to be faster. A monolayer of fibroblast cells commenced to grow. In the third and fourth subcultures the schizont-infected cells increased to $3.0 \times 10^5$ and to $5.0 \times 10^5$ cells per ml. In further passages the infected cells increased in numbers to about $5.0 \times 10^5$ cells per ml after cultivation for four days. The stained coverslips showed large monocytes or blast cells containing schizonts. No schizonts were observed in the fibroblasts. Isolation of schizont-infected cells was not possible from each individual test tube, containing 0.5 ml or one ml blood but, on the fourth subculture, the cells which had survived in five test tubes were pipetted off and pooled in a two ounce bottle. This culture was then established in five weeks of maintenance and subculturing. Two out of three cultures in the Carrel flasks established successfully in three weeks time (see Table 4.1).

Experiment 4.3

Isolation of strain 3 (S.3).

An eight months old, male, Holstein calf No. 4079 inoculated

with T. annulata (S.3) was used. The body temperature was 40.3°C on day 13 after inoculation. The stained smears of the lymph node contained five to ten schizonts per microscopic field but no schizonts were observed in the blood films.

Jugular venous blood was taken from the calf and citrated and defibrinated blood was separately prepared as described before. The methods used in this experiment were similar to those in Experiments 4.1 and 4.2, with some modifications.

(a) The citrated blood was treated with ammonium chloride solution to separate the white blood cells as described before. The numbers of WBC were adjusted to $1.0 \times 10^6$ and $2.0 \times 10^6$ per ml of medium for cultivation. They were distributed into two ounce medical flats and incubated at 37°C. Subcultures were made every three to five days according to the growth rate.

(b) The leucocytes were separated from the citrated blood by lysing the rbc using distilled water. This was done according to the technique described by Odajima and Sonoda (1970). The separated leucocytes were treated and cultured as in trial (a).

(c) In this trial, aliquots of ten ml and 20 ml defibrinated whole blood were dispensed into the Carrel flasks and into 200 ml medical flats. The culture vessels were set up in the incubator in an inclined position, some transversely and some longitudinally, so that the upper edge of the blood was half way up the slope. After three days the blood suspensions were carefully drawn out by pasteur pipette from the bottom of the vessels. The cultures were then washed out gently with medium and the cells which had adhered to the surfaces remained. Growth medium was added to the cultures and they were incubated. From the third to the fifth passages the cultures were brought to suspension by pipetting and

agitating the cells which had adhered to the surfaces. Successive, twice weekly subcultures with about $2.0 \times 10^5$ cells per ml were carried out until stability of the cultures was achieved.

RESULTS

The schizont-infected cells in trials (a) and (c) established after five passages, in 18 and 20 days respectively. The infected cells in trial (b), which were separated using distilled water, were also established but in four weeks time, (Table 4.1).

Experiment 4.4

Isolation of strain 15 (S.15) using citrated and defibrinated blood.

In this experiment a male Holstein calf No. 104, eight months old, was inoculated with T. annulata, S.15. The body temperature of the animal was recorded as 40.1°C on the 13th day after inoculation. The lymph node biopsy smears showed one to five schizonts per microscopic field and free schizonts but the parasites were not detectable in blood smears.

Citrated blood was used for the production of suspensions of white cells by lysis of the red cells with ammonium chloride and the defibrinated blood was used whole to separate white cells by the process of adhesion, the methods being as described in the previous experiments.

(a) The final suspension of the leucocytes was adjusted to $1.0 \times 10^6$ and $2.0 \times 10^6$ per ml of medium and dispensed into two ounce medical flats which were incubated and maintained as described in the experiment 4.1.

(b) Aliquots of ten and 20 ml of defibrinated blood were

distributed in Carrel flasks and 200 ml medical flats. These culture vessels were incubated for three days, as explained in Experiment 4.2.

RESULTS

The cultures of series (a) within the first two passages showed many dead cells. In the cultures initiated with $1.0 \times 10^6$ cells per ml only a few schizont-infected cells survived, and these died off within twelve days. The cultures initiated with $2.0 \times 10^6$ leucocytes per ml produced a large number of viable cells. These cells, which were pooled from two medical flats, established well in the next two passages, (16 days). The schizont-infected lymphocytes in the series (b) showed high efficiency in adhering to the surface and were not readily pipetted off. The Theileria-infected cells were established after four successive subcultures in 16 days (Table 4.1).

Experiment 4.5

Isolation of strain 19 (S.19).

In this experiment attempts were made to isolate schizont-infected cells from infected blood at the various stages of the disease.

A male, eight months old, Holstein calf No. 4050, which was inoculated with *T. annulata*, (S.19) was used.

(a) On day 17 after inoculation blood was drawn when the body temperature of the calf was 40.0°C, the lymph node smears demonstrated one schizont per microscopic field and no schizonts were observed in blood smears. The intra-erythrocytic form was detected at the rate of one per 1000 rbc.

(b) Blood was drawn on the 19th day after inoculation when the animal was showing a temperature of 41.6°C and the schizonts in the biopsy smears of the prescapular node and the liver were as many as five to ten per microscopic field. No schizonts were seen in blood smears. The number of the intra-erythrocytic forms was increased to 80 per 1000 rbc.

(c) Blood was drawn on day 25 after inoculation, when the calf's body temperature fell to the normal 38.8°C. At this stage, no schizonts were observed in lymph node smears or blood smears. Infection of the red blood cells with the intra-erythrocytic form was at the rate of 150 per 1000 rbc.

Attempts to isolate the parasite were made using ammonium chloride lysis and defibrination as described in Experiment 4.3.

RESULTS

In the trials (a) and (b), the early and middle stages of the disease produced schizont-infected cells, established from the cultures seeded with 2.0 x $10^6$ leucocytes per ml after lysis of red cells after 24 and 21 days, respectively. In trial (c) the late or recovered stage did not yield schizont-infected cells from the leucocyte suspension cultures. No positive cultures were produced by using whole blood in any of the three trials (Table 4.1).

Experiment 4.6

Isolation of strain 19 (S.19).

In this experiment an attempt was made to determine the cause of the failure to isolate strain 19 from whole blood in the previous experiment. X how?

A male Holstein calf No. 3968, six months old, was inoculated

with T. annulata, S.19. Nineteen days after inoculation, when the calf was bled, the body temperature was recorded as 41.5°C, the lymph node biopsy smears showed ten to 15 schizonts per microscopic field and these were frequent in peripheral blood. The infection rate of the red blood cells with the intra-erythrocytic forms was 240 per 1000 rbc. Samples of leucocyte suspension and of whole blood were used for attempted isolation as before.

RESULTS

The leucocyte suspension cultures showed a sharp decline of viable cells in the first two days. Those which survived grew only slowly. After twelve days, there was a marked increase in the numbers of the viable cells. In the cultures of the whole blood the schizont-infected cells underwent the same process of maintenance for three passages. In the fourth and fifth subcultures, establishment occurred at 14 and 18 days respectively, (Table 4.1).

Experiment 4.7

Isolation of strain 20 (S.20).

A five months old, male Holstein calf No. 3975 was inoculated with T. annulata, S.20 and used for two trials as follows:-

(a) Eighteen days after inoculation, when the body temperature was 40.0°C, ten to 15 schizonts were seen in the lymph node smears and none in the blood film. Intra-erythrocytic forms were detected at the rate of one per 1000 rbc in blood smears.

(b) On the following day, the body temperature was 39.9°C, one to five schizonts were seen per microscopic field in lymph node smears,

and in blood smears schizonts were detectable but rare. Five per 1000 rbc were infected with piroplasms. The blood samples were prepared and isolation of the schizont-infected cells was attempted as described for the two previous experiments.

RESULTS

The schizont-infected cells in both trials were readily established. In trial (a) establishment was achieved in 20 days and in trial (b) in 16 days (Table 4.1).

Experiment 4.8

Isolation of strain 21 (S.21).

For this experiment, T. annulata, S.21 was inoculated into a male Holstein calf No. 3962 aged ten months. On the 19th day after inoculation the body temperature was 40.9°C, schizonts were present in lymph node smears (ten to 15 per microscopic field) but were not detectable in blood smears. Intra-erythrocytic forms at the rate of one per 1000 rbc were present. The calf was bled at this stage and the blood samples were treated for the isolation as before.

RESULTS

The schizont-infected cells were established in three weeks time. The details of this and the previous seven experiments are tabulated in Table 4.1.

II. Isolation of T. annulata strains from infected lymph nodes in suspension cultures.

Experiments 4.9 to 4.13

Isolation of strains, S.3, S.15, S.19, S.20 and S.21.

Table 4.1

Isolation of various strains of *T. annulata*-infected lymphoid cells from infected blood.

| Experiment No. | Strain | Calf No. | Age (months) | Post inoculation (days) | Body temperature (°C) | No. of schizonts/ mic. field (lymph node) | Schizonts in blood smear | No. of piroplasms/ 1000 rbc | Culture method | | No. of cultures tried | No. of positive cases | Time for establishment (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Suspended WBC/ ml. $\times 10^6$ | Whole blood (ml.) | | | |
| 4.1 | 3 | 2539 | 12 | 18 | 40.6 | 10-15 | (++) | (-) | 0.5 1.0,2.0 | | 6 | 4 | 16,20 |
| 4.2 | 3 | 2539 | 12 | 20 | 41.0 | 10-15 | (+++) | (-) | | 0.5, 1.0, 10.0 | 13 | 3 | 35 21 |
| 4.3 (a), (b), (c) | 3 | 4079 | 8 | 13 | 40.3 | 5-10 | (-) | (-) | 1.0, 2.0 | 10.0, 20.0 | 6 | 6 | 18,(a) 28,(b) 20 (c) |
| 4.4 (a) (b) | 15 | 104 | 8 | 13 | 40.1 | 1-5 | (-) | (-) | 1.0, 2.0 | 10.0, 20.0 | 6 | 4 | 16 (a) 16 (b) |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4.5 (a) | 19 | 4050 | 8 | 17 | 40.0 | 1 | (-) | 1 | 1.0, 2.0 | 10.0, 20.0 | 6 | 2 | 24 |
| 4.5 (b) | 19 | 4050 | 8 | 19 | 41.6 | 5-10 | (-) | 80 | 1.0, 2.0 | 10.0, 20.0 | 6 | 2 | 21 |
| 4.5 (c) | 19 | 4050 | 8 | 25 | 38.8 | 0 | (-) | 150 | 1.0, 2.0 | 10.0, 20.0 | 6 | 0 | |
| 4.6 | 19 | 3968 | 6 | 19 | 41.5 | 10-15 | (++) | 240 | 1.0, 2.0 | 10.0, 20.0 | 6 | 6 | 14 18 |
| 4.7 (a) | 20 | 3975 | 5 | 18 | 40.0 | 10-15 | (-) | 1 | 1.0, 2.0 | 10.0, 20.0 | 6 | 6 | 20 |
| 4.7 (b) | 20 | 3975 | 5 | 19 | 39.9 | 1-5 | (+) | 5 | 1.0, 2.0 | 10.0, 20.0 | 6 | 6 | 16 |
| 4.8 | 21 | 3962 | 10 | 19 | 40.9 | 10-15 | (-) | 1 | 1.0, 2.0 | 10.0, 20.0 | 6 | 6 | 21 |

(-) = not detected (+) = rare (++) = frequent (+++) = very frequent

Five successful isolations were carried out from lymph node biopsy materials of the infected calves. The lymph node samples were prepared when the animals were reacting to the disease and the lymph node smears showed schizonts. The lymph node tissue obtained by biopsy, as described in Chapter 3, was transferred into a test tube containing growth medium. The tissue was pipetted to disintegrate the schizont-infected cells. This was then centrifuged at 160 G for seven minutes. The supernatant fluid was discarded and the cells and accompanying tissue fragments were distributed in two ounce medical flats. The cultures were checked and stained smears of the cells were examined daily. They were subcultured twice weekly, adjusting the number of the cells to not less than $5.0 \times 10^5$ cells per ml of medium.

RESULTS

Stained culture smears showed blastoid cells containing schizonts varying in size and number. In the first two days there was some decrease in the number of schizont-infected lymphoid cells but thereafter, the numbers of viable cells increased. In twelve to 16 days all five strains were established and continued their growth *in vitro*. The data of these experiments are tabulated in Table 4.2.

III. Isolation from infected liver.

Experiment 4.14

Isolation of strain 19 (S.19).

Liver biopsy was performed as described in Chapter 3, and the liver tissue was used for isolation. Calf No. 4050, 19 days after inoculation, was used for this experiment. The calf showed five

Table 4.2

Isolation of various strains of *T. annulata*-infected lymphoid cells from lymph node biopsy.

| Experiment No. | Strain | Calf No. | Age (months) | Post inoculation (days) | Body temperature (°C) | No. of schizonts /mic. field (lymph node) | Schizonts in blood smear | No. of piroplasms /1000 rbc | Approx. No. of suspended cells/ ml, $\times 10^6$ | No. of cultures tried | No. of positive cases | Time for establishment (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4.9 | 3 | 2539 | 12 | 18 | 41.5 | 10 - 15 | (++) | (-) | 1.0 | 4 | 3 | 16 |
| 4.10 | 15 | 2531 | 15 | 24 | 40.2 | 5 - 10 | (-) | (-) | 1.0 | 2 | 2 | 12 |
| 4.11 | 19 | 3968 | 6 | 19 | 41.5 | 10 - 15 | (+) | 140 | 1.0 | 3 | 2 | 15 |
| 4.12 | 20 | 3975 | 5 | 18 | 41.0 | 10 - 15 | (-) | 1 | 1.0 | 2 | 2 | 14 |
| 4.13 | 21 | 3962 | 10 | 19 | 40.0 | 10 - 15 | (-) | 1 | 1.0 | 2 | 2 | 14 |

(-) = not detected, (+) = rare, (++) = frequent.

to ten schizonts per microscopic field in both liver smears and lymph node smears and a temperature of 41.6°C. In blood smears no schizonts were detected but piroplasms were assessed at the rate of 80 per 1000 rbc. The liver tissues after pipetting, washing and centrifugation, were resuspended in the growth medium in several two ounce medical flats. These were also subcultured twice weekly until the infected cells were established.

RESULTS

The schizont-infected cells were successfully established in four subcultures, after 14 days. The data are tabulated in Table 4.3.

B. From autopsy materials.

I. Isolation from infected lymph node, (II) from spleen and (III) from liver.

Experiment 4.15

Isolation of strain 3 (S.3).

The Holstein calf No. 2539, male and twelve months old, which was inoculated with <u>T. annulata</u> (S.3), showed typical symptoms of theileriosis and eventually died 23 days after inoculation. For isolation of schizont-infected cells, three samples from: (I) lymph node, (II) spleen and (III) liver tissues were taken. The cells were prepared from these infected tissues as described in Chapter 3. These cells were separately pipetted, washed and centrifuged as before and finally resuspended in the medium, in two and four ounce medical flats. Numbers of the infected cells were adjusted to approximately $1.0 \times 10^6$ per ml culture and they were passaged twice weekly.

Table 4.3

Isolation of *T. annulata*-infected lymphoid cells from liver biopsy.

| Experiment No. | Strain | Calf No. | Age (months) | Post-inoculation (days) | Body temperature ($^oC$) | No. of schizonts /mic. field | | Schizonts in blood smear | No. of piroplasms /1000 rbc | Approx. No. of suspended cells/ml x $10^6$ | No. of cultures tried | No. of positive cases | Time for establishment (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | (lymph node) | (liver) | | | | | | |
| 4.14 | 19 | 4050 | 8 | 19 | 41.6 | 5-10 | 5-10 | (-) | 80 | 1.0 | 2 | 2 | 14 |

(-) = not detected.

RESULTS

Daily examination of the cultures showed that spleen and liver implants did not establish due to necrosis of the cells or because of heavy bacterial contamination. The schizont-infected cells from lymph node, however, established after five subcultures, in 18 days. The data are shown in Table 4.4.

Experiment 4.16

Isolation of strain 19 (S.19).

The Holstein calf No. 3968, male and six months old, which had been infected with T. annulata (S.19), was used. The calf died 21 days after the inoculation, showing all the symptoms of theileriosis. Attempts were made to isolate schizont-infected cells from: (I) lymph node, (II) spleen and (III) liver, as in the previous experiment.

RESULTS

All three trials resulted in successful isolation as is shown in Table 4.4.

DISCUSSION

Theileria annulata can readily be transmitted to susceptible animals by the inoculation of blood from an infected animal at the early and middle stages of the disease, even when the schizonts are not discernible in blood smears, (Sergent et al 1945 and Neitz 1957). Tsur and Adler (1965) isolated T. annulata from blood of infected cattle when no schizonts were detected in blood smears. These authors used coagulated buffy coat which was then trypsinised and cultivated in monolayer. Hulliger (1965) isolated

Table 4.4

Isolation of two strains of _T. annulata_-infected lymphoid cells from autopsy materials.

| Experiment No. | Strain | Calf No. | Age (months) | Post-inoculation (days) | No. of schizonts/mic. field | | | | No. of piroplasms /1000 rbc | Tissues used of approx. $1 \times 10^6$ cells/ml medium | No. of cultures tried | No. of positive cases | Time for establishment (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | in lymph node | in spleen | in liver | in blood | | | | | |
| 4.15 (I) | 3 | 2539 | 12 | 23 | >15 | >15 | 10-15 | (+++) | (-) | lymph node | 3 | 1 | 18 |
| 4.15 (II) | 3 | 2539 | 12 | 23 | >15 | >15 | 10-15 | (+++) | (-) | spleen | 3 | 0 | |
| 4.15 (III) | 3 | 2539 | 12 | 23 | >15 | >15 | 10-15 | (+++) | (-) | liver | 3 | 0 | |
| 4.16 (I) | 19 | 3968 | 6 | 21 | 10-15 | >15 | 10-15 | (++) | 250 | lymph node | 3 | 3 | 16 |
| 4.16 (II) | 19 | 3968 | 6 | 21 | 10-15 | >15 | 10-15 | (++) | 250 | spleen | 3 | 2 | 14 |
| 4.16 (III) | 19 | 3968 | 6 | 21 | 10-15 | >15 | 10-15 | (++) | 250 | liver | 3 | 2 | 14 |

(-) = not detected, (+) = rare, (++) = frequent, (+++) = very frequent.

T. annulata from buffy coat prepared from blood of infected animals in association with BHK cells. This author stated that association of BHK cells or fibroblast cells was necessary for continuous growth of infected lymphoid cells. The feeder cell layer is generally thought to facilitate establishment and growth of schizont-infected cells but it has certain disadvantages in that, firstly Theileria-infected cells are mixed with other cells and secondly that this admixture makes the percentage of infected cells difficult to determine. For these reasons attempts were made to establish the cultures in suspension form throughout all the isolations described here. In isolation of schizont-infected cells from the blood, two simple methods were used. One by lysing rbc and separating WBC either with ammonium chloride solution or with distilled water, (Odajima and Sonoda 1970 and 1971). The other, using whole blood either defibrinated or citrated, utilising the adhesion property of the infected leucocytes, (Tokuda et al 1962). The schizont-infected cells which were separated by lysis of the rbc with distilled water established themselves more slowly than did those in which ammonium chloride was used. Observations indicated that the use of ammonium chloride did not have any more deleterious effect on the WBC than did distilled water. Therefore, ammonium chloride lysis was used for the separation of WBC from the blood thereafter. It was also seen that there was no discernible difference between citrated and defibrinated blood in this process. Usage of whole blood represented a new and simple method of isolation of T. annulata from the infected animal, thus obviating the necessity for more complicated procedures such as lymph node and liver biopsy and other methods of processing blood. Success in making the first

isolation of the very virulent S.3, in suspension culture from blood was achieved by using a high seeding rate ($1.0 \times 10^6$ and $2.0 \times 10^6$ cells per ml) with no feeder layers (Experiment 4.1). The observations in Experiments 4.2 and 4.3 showed that schizont-infected cells (S.3) had a high adhesion efficiency which brought about adhesion of transformed lymphoblasts in successful isolation. The addition of lactalbumin hydrolysate and yeast extract to Eagle's medium (see Chapter 3) was shown to have a definitely beneficial effect on isolation and growth of schizont-infected cells, thus confirming previous work, (Hooshmand-Rad 1973 and 1975; Hashemi-Fesharki and Shad-Del 1973a and 1973b). It was observed that when the schizonts were detected in blood smears and especially in considerable numbers, their establishment was easier and more rapid. The opposite case was less successful in isolation (Experiment 4.4). The failure to isolate S.19 (Experiment 4.5c) was thought to be due to the nature of the strain. This was a wild one which produced erythrocytic forms but it was proved in the following Experiment 4.6 that the difficulty in isolation was due to insufficiency of the infected leucocytes in blood samples, as the same strain (S.19) was readily isolated in this experiment whichever method was used. The virulence of the strains appeared to affect isolation, the less virulent the strain, the easier the isolation. This supports the observations of Hooshmand-Rad and Hashemi-Fesharki (1968). Two wild strains, (S.20 and S.21) which had many characteristics in common, were milder than S.3 and both established with ease (Experiments 4.7 and 4.8).

Isolation of schizont-infected cells from biopsy materials of lymph node and liver were achieved more easily and rapidly

than was isolation from infected blood, because schizont-infected cells were plentiful. These were dispersed from the tissues only by pipetting, without any trypsinisation (Experiments 4.9 to 4.14).

Isolation of schizont-infected cells from autopsy materials, lymph node, spleen and liver were successful only when the samples were taken shortly after the infected animal died (Experiments 4.15 and 4.16).

# CHAPTER 5

## CULTIVATION OF T. ANNULATA-INFECTED LYMPHOID CELLS IN SUSPENSION

Cultivation of schizont-infected cells of various strains of T. annulata in various growth media was studied. Different factors affecting the growth rate of schizont-infected cells were investigated. Following the isolation and establishment of five strains of T. annulata, S.3, S.15, S.19, S.20 and S.21, as described in Chapter 4, growth rates of schizont-infected lymphoid cells of these strains were compared.

### Experiment 5.1

Difference of the growth rates of various strains of T. annulata.

The growth medium used in this experiment was modified Eagle's medium (1955) with slight variation in amount of some ingredients as adopted by Hooshmand-Rad (1973) and Hashemi-Fesharki and Shad-Del (1973a and 1973b). This was supplemented with ten per cent calf serum (CS), 100 mg per cent w/v lactalbumin hydrolysate and 20 mg per cent w/v yeast extract (LY) in the final volume. The pH of the medium was adjusted to 7.2 using sodium bicarbonate. The numbers of viable schizont-infected cells from each strain were adjusted to $1.0 \times 10^5$ per ml of medium. The cultures were set up in 100 ml medical flats and incubated at 37°C for five days. They were sampled and duplicate cell counts were carried out daily in three successive cultures.

RESULTS

The growth rates of the cells infected with these strains are tabulated in Table 5.1 and are shown in Fig. 5.1. In the three cultures of S.3, the mean increase of the cells implanted was 5.35 fold in four days. The corresponding increases for S.15, S.19, S.20 and S.21 were 10.16, 5.48, 7.02 and 7.60, respectively. In the first 24 hours, although a general increase was observed, this was fairly small. The cells multiplied in a sigmoid manner from one to four days after cultivation. The growth then slowed down and there was a reduction in numbers of viable cells.

Experiment 5.2

Attempts to cultivate schizont-infected cells in some simple growth media.

In the following experiments schizont-infected cells of S.15 were used in studying various parameters, unless otherwise stated.

Three simple media were prepared for the cultivation of schizont-infected cells as follows:-

(a) Phosphate buffered saline (PBS) without calcium and magnesium salts but with ten per cent v/v CS and 0.5 per cent w/v lactalbumin hydrolysate and 0.1 per cent w/v yeast extract (LY). The pH of the medium was adjusted to 7.2, using sodium bicarbonate.

(b) Similarly prepared but with the PBS replaced by Hank's balanced salt solution.

(c) Earle's balanced salt solution with the same supplements as in (a) and (b). To all the growth media were added penicillin and streptomycin (PS) at the rate of 100 units and 100 μg per ml of medium respectively. The growth medium used for the control cultures was modified Eagle's, containing the same percentages of

Table 5.1

Difference of growth rate of lymphoid cells infected with various strains of _T. annulata_, cultivated in modified Eagle's medium supplemented with CS and LY.

| Strains | Cells/ml | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| 3 | M. | 100.0 | 158.3 | 339.7 | 497.0 | 535.0 | 459.0 |
| | S.D. | | 29.0 | 47.6 | 15.7 | 61.4 | 43.9 |
| 15 | M. | 100.0 | 178.3 | 356.3 | 621.3 | 1016.7 | 888.3 |
| | S.D. | | 39.4 | 47.5 | 29.0 | 27.5 | 24.4 |
| 19 | M. | 100.0 | 107.3 | 222.7 | 407.3 | 548.0 | 497.7 |
| | S.D. | | 17.8 | 15.4 | 35.2 | 79.8 | 41.5 |
| 20 | M. | 100.0 | 147.7 | 323.0 | 529.0 | 702.0 | 633.0 |
| | S.D. | | 17.5 | 47.5 | 79.5 | 39.0 | 53.1 |
| 21 | M. | 100.0 | 169.7 | 363.0 | 558.7 | 760.0 | 638.0 |
| | S.D. | | 21.8 | 36.3 | 72.5 | 54.1 | 52.0 |

Mean numbers of cells per ml, counted daily from each strain, $x10^3$

M = Mean cell counts of three subcultures    S.D.= Standard Deviation

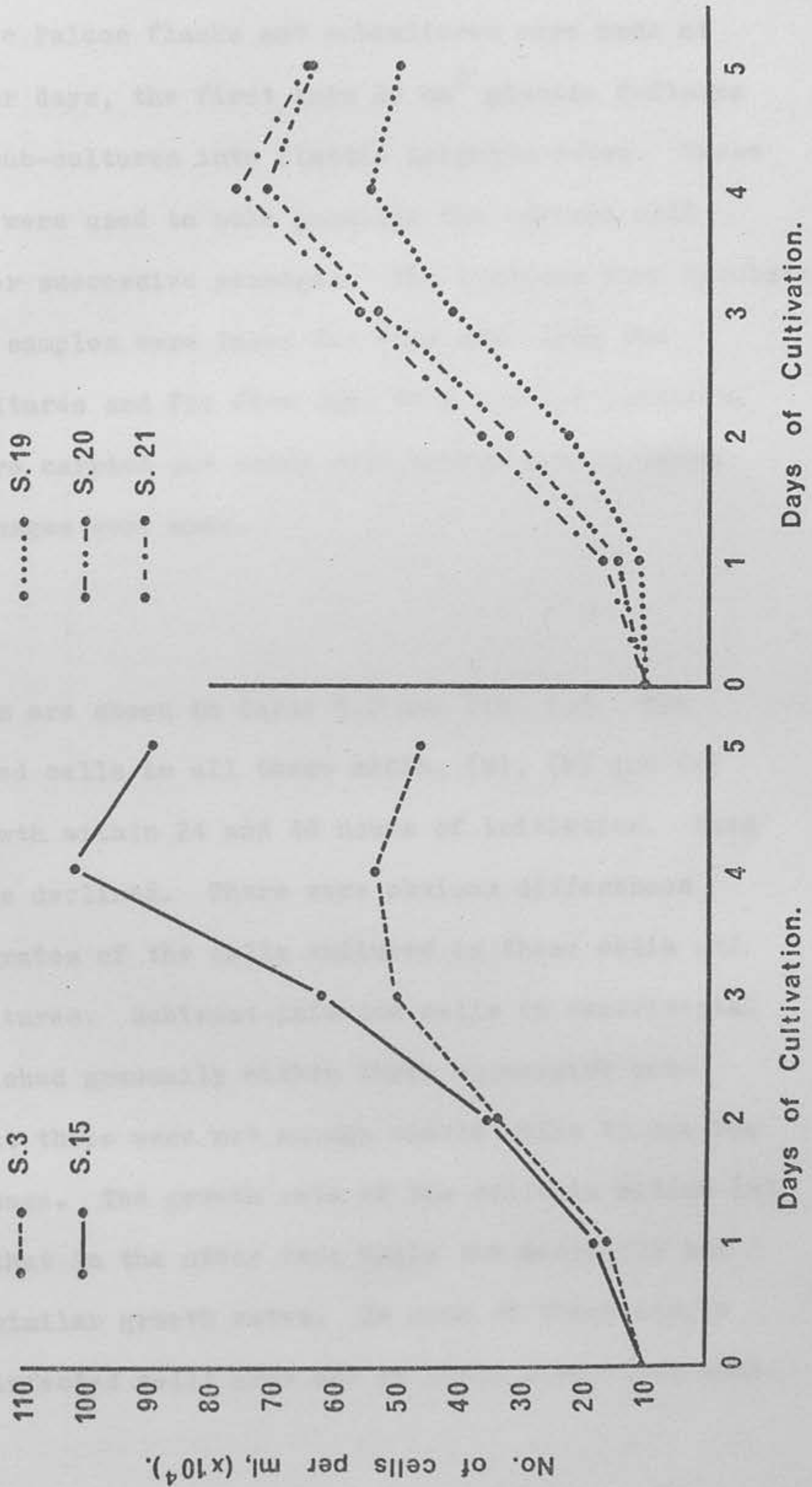



Fig. 5.1 Difference of growth rate of lymphoid cells infected with various strains of T. annulata, cultivated in modified Eagle's medium. Values are means of three subcultures.

CS, LY and PS as in the above media. Each culture was initiated with $1.0 \times 10^5$ viable schizont-infected cells per ml of medium in 75 $cm^2$ plastic Falcon flasks and subcultures were made at intervals of four days, the first into 25 $cm^2$ plastic T-flasks and subsequent sub-cultures into plastic Leighton tubes. These culture vessels were used to make possible the correct cell concentration for successive passages. The cultures were incubated at 37°C. Daily samples were taken for four days from the experimental cultures and for five days from control cultures. Three trials were carried out using each medium and in every trial three passages were made.

RESULTS

The results are shown in Table 5.2 and Fig. 5.2. The schizont-infected cells in all three media, (a), (b) and (c) showed some growth within 24 and 48 hours of initiation. Then the growth rates declined. There were obvious differences between growth rates of the cells cultured in these media and the control cultures. Schizont-infected cells in experimental cultures diminished gradually within three successive sub-cultures so that there were not enough viable cells to use for the fourth passage. The growth rate of the cells in medium (a) was less than that in the other two; while the media (b) and (c) supported similar growth rates. In none of these simple media did the infected cells grow and maintain themselves well.

Table 5.2

Attempts to cultivate schizont-infected cells in some simple media.

| Growth Media | Cul-ture | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| (a) PBS plus supplements | 1 | 100.0 | 109.0 | 165.0 | 106.7 | 86.7 | |
| | 2 | 100.0 | 115.0 | 106.7 | 73.3 | 36.7 | |
| | 3 | 100.0 | 63.3 | 55.0 | 25.0 | 8.3 | |
| (b) Hank's salts plus supplements | 1 | 100.0 | 101.7 | 136.7 | 76.7 | 55.0 | |
| | 2 | 100.0 | 111.7 | 136.7 | 71.7 | 33.3 | |
| | 3 | 100.0 | 85.0 | 91.7 | 51.7 | 21.7 | |
| (c) Earle's salts plus supplements | 1 | 100.0 | 140.0 | 195.0 | 131.7 | 90.0 | |
| | 2 | 100.0 | 111.7 | 125.0 | 85.0 | 45.0 | |
| | 3 | 100.0 | 106.7 | 85.0 | 45.0 | 13.3 | |
| (d) Control, Modified Eagle's Medium | 1 | 100.0 | 155.0 | 370.0 | 680.0 | 1005.0 | 890.0 |
| | 2 | 100.0 | 130.0 | 325.0 | 650.0 | 955.0 | 820.0 |
| | 3 | 100.0 | 180.0 | 405.0 | 715.0 | 900.0 | 765.0 |

No. of viable cells per ml, $x10^3$

The figures in each culture period represent mean values, obtained from three trials for each medium.

Experiment 5.3

Comparison of growth rate of schizont-infected cells cultivated in various standard media.

There are a number of growth media which have been used to grow Theileria-infected cells. Four of these media prepared as described in Chapter 3 were compared in this experiment to evaluate which one would be most suitable for the growth of T. annulata. The media used in this comparison were as follows:-

(a) Modified Eagle's medium prepared according to Hooshmand-Rad (1973).

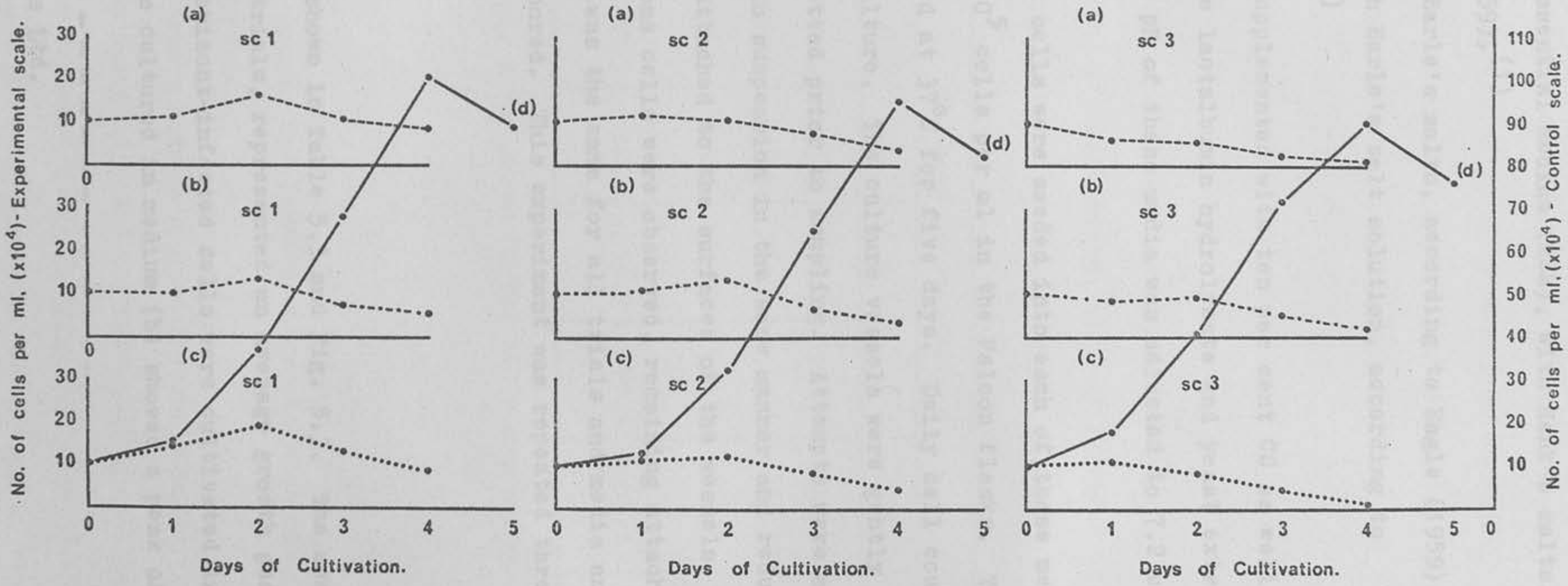



Fig. 5·2 Growth rate of schizont-infected cells in some simple media and modified Eagle's medium. (a) PBS salts plus supplements (b) Hank's salts plus supplements (c) Earle's salts plus supplements (d) modified Eagle's medium, control.

(b) Eagle's minimum essential medium (MEM), with Hank's salts, according to Eagle (1959).[1]

(c) Eagle's MEM with Earle's salts, according to Eagle (1959).[1]

(d) TC Medium 199 with Earle's salt solution, according to Morgan *et al* (1950).[2]

All these media were supplemented with ten per cent CS as well as 1.0g and 0.2g per litre lactalbumin hydrolysate and yeast extract LY, respectively. The pH of these media was adjusted to 7.2 using sodium bicarbonate.

Schizont-infected cells were seeded into each of these media at the rate of 1.0 x $10^5$ cells per ml in the Falcon flasks. The cultures were incubated at 37°C for five days. Daily cell counts were made from each culture. The culture vessels were gently and evenly shaken and pipetted prior to sampling. Attempts were made to bring the cells into suspension in the same manner and reduce the numbers of cells attached to the surfaces of the vessels. It is admitted that some cells were observed, remaining attached to the surface. This was the same for all trials and media and therefore could be ignored. This experiment was repeated three times.

RESULTS

The results are shown in Table 5.3 and Fig. 5.3. The mean cell counts of three trials, represented an average growth peak of 10.98 fold, when schizont-infected cells were cultivated in the medium (a). The cells cultured in medium (b) showed a peak of

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(1) Flow Laboratories Ltd.

(2) Wellcome Reagents Ltd.

9.46 fold. (c) In this medium, the maximum growth rose to 16.26 fold. (d) The infected cells in this medium showed a 15.53 fold increase. In all the media the peak of the cell growth occurred on the fourth day of cultivation and thereafter the decline started.

Table 5.3

Comparison of growth rate of schizont-infected cells cultivated in various standard media, all supplemented with CS and LY.

| Growth media | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| (a) Modified Eagle's | 100.0 | 125.0 | 315.0 | 598.3 | 1098.3 | 925.0 |
| (b) TC Medium 199 | 100.0 | 113.3 | 298.3 | 560.0 | 946.7 | 871.7 |
| (c) Eagle's MEM with Hank's | 100.0 | 183.3 | 410.0 | 730.0 | 1626.7 | 1208.3 |
| (d) Eagle's MEM with Earle's | 100.0 | 136.7 | 316.7 | 668.3 | 1553.3 | 1323.3 |

No. of cells per ml, x $10^3$ shown in the table are the means of three trials.

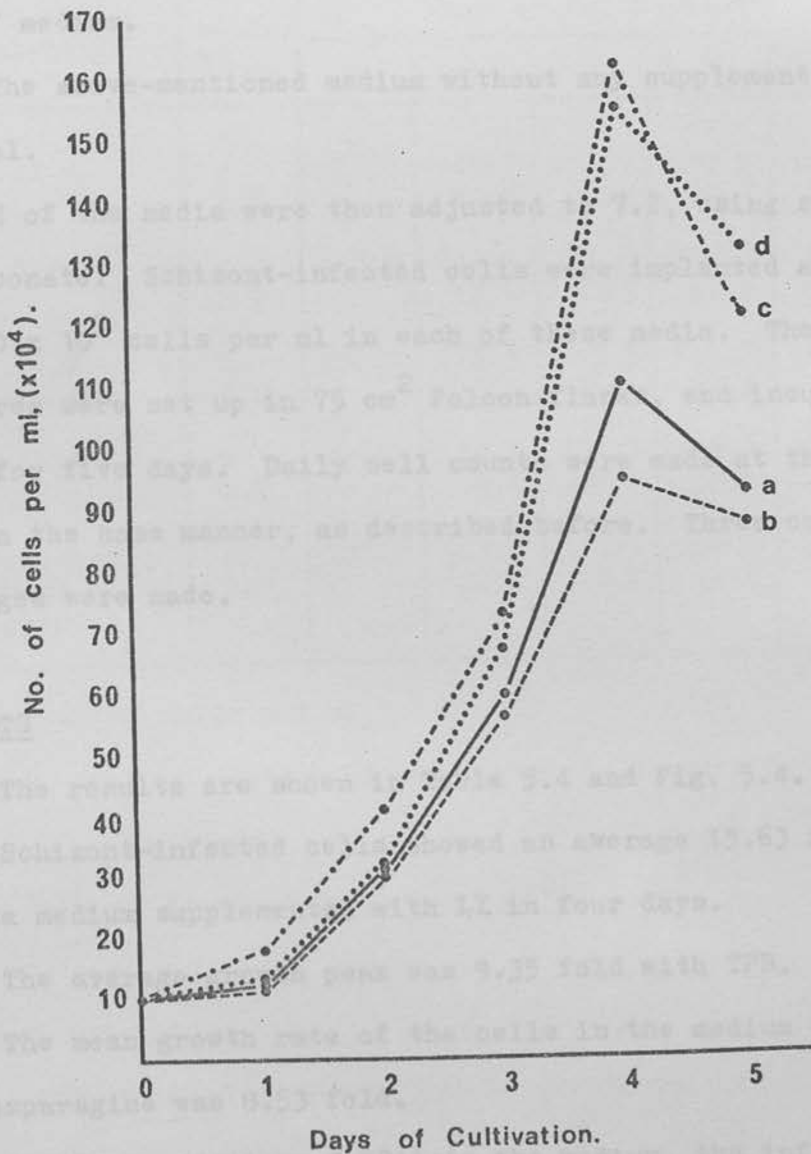
Experiment 5.4

Effect of various growth supplements on growth rate of schizont-infected cells.

A number of supplements have been used in growth media for improving the growth of lymphoid cells infected with Theileria spp. To examine which one is preferable for T. annulata-infected cells, a batch of 500 ml Eagle's MEM with Hank's salts containing ten per

Fig. 5·3 Comparison of growth rate of schizont-infected cells cultivated in various media, all supplemented with CS and LY.

- a ——— modified Eagle's (1955)
- b - - - - TC medium 199
- c -·-·- Eagle's MEM (1959) with Hank's salts
- d ······ Eagle's MEM (1959) with Earle's salts



cent v/v calf serum was made. This was dispensed into five equal volumes and the following supplements were added to four of them; and the fifth one was used without supplements, as a control:-

(a) Lactalbumin hydrolysate and yeast extract (LY) at the rate of 100mg and 20mg per cent w/v, respectively.

(b) Tryptose phosphate broth (TPB) which was added ten per cent v/v from a two per cent w/v stock solution.

(c) L-$\beta$ asparagine at the rate of ten mg per cent v/v of medium.

(d) Non-essential amino acids (NEAA) at the rate of one per cent v/v of medium.

(e) The above-mentioned medium without any supplement, as a control.

The pH of the media were then adjusted to 7.2, using sodium bicarbonate. Schizont-infected cells were implanted at the rate of $1.0 \times 10^5$ cells per ml in each of these media. The cell cultures were set up in 75 $cm^2$ Falcon flasks, and incubated at $37^oC$ for five days. Daily cell counts were made at the same time and in the same manner, as described before. Three consecutive passages were made.

RESULTS

The results are shown in Table 5.4 and Fig. 5.4.

(a) Schizont-infected cells showed an average 15.63 fold increase in the medium supplemented with LY in four days.

(b) The average growth peak was 9.35 fold with TPB.

(c) The mean growth rate of the cells in the medium containing L-$\beta$ asparagine was 8.53 fold.

(d) With the addition of NEAA to the medium, the infected cells gained a 10.16 fold increase.

(e) Although schizont-infected cells grew readily in the medium without any supplement, the peak of growth after four days was 7.75 fold of the initial number of implanted cells.

Table 5.4

Growth rate of schizont-infected cells cultivated in Eagle's MEM with Hank's salts and CS and with different supplements.

| Supplements | cells/ml | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| (a) LY | M | 100.0 | 146.7 | 296.7 | 690.0 | 1563.3 | 1228.3 |
| | S.D. | | 12.6 | 27.5 | 57.7 | 193.2 | 97.1 |
| (b) TPB | M | 100.0 | 138.3 | 325.0 | 548.3 | 935.0 | 831.7 |
| | S.D. | | 12.6 | 87.9 | 37.9 | 195.8 | 106.1 |
| (c) L-$\beta$ asparagine | M | 100.0 | 118.3 | 248.3 | 426.7 | 853.3 | 750.0 |
| | S.D. | | 35.1 | 76.5 | 77.5 | 135.0 | 81.8 |
| (d) NEAA | M | 100.0 | 118.3 | 231.7 | 416.7 | 1016.7 | 940.0 |
| | S.D. | | 38.8 | 58.4 | 120.9 | 210.8 | 153.9 |
| (e) Control, without supplement | M | 100.0 | 105.0 | 158.3 | 348.3 | 775.0 | 535.0 |
| | S.D. | | 22.9 | 22.5 | 46.5 | 86.7 | 65.4 |

Nos. of cells per ml, x $10^3$

M = Mean numbers of cells per ml, counted from three cultures

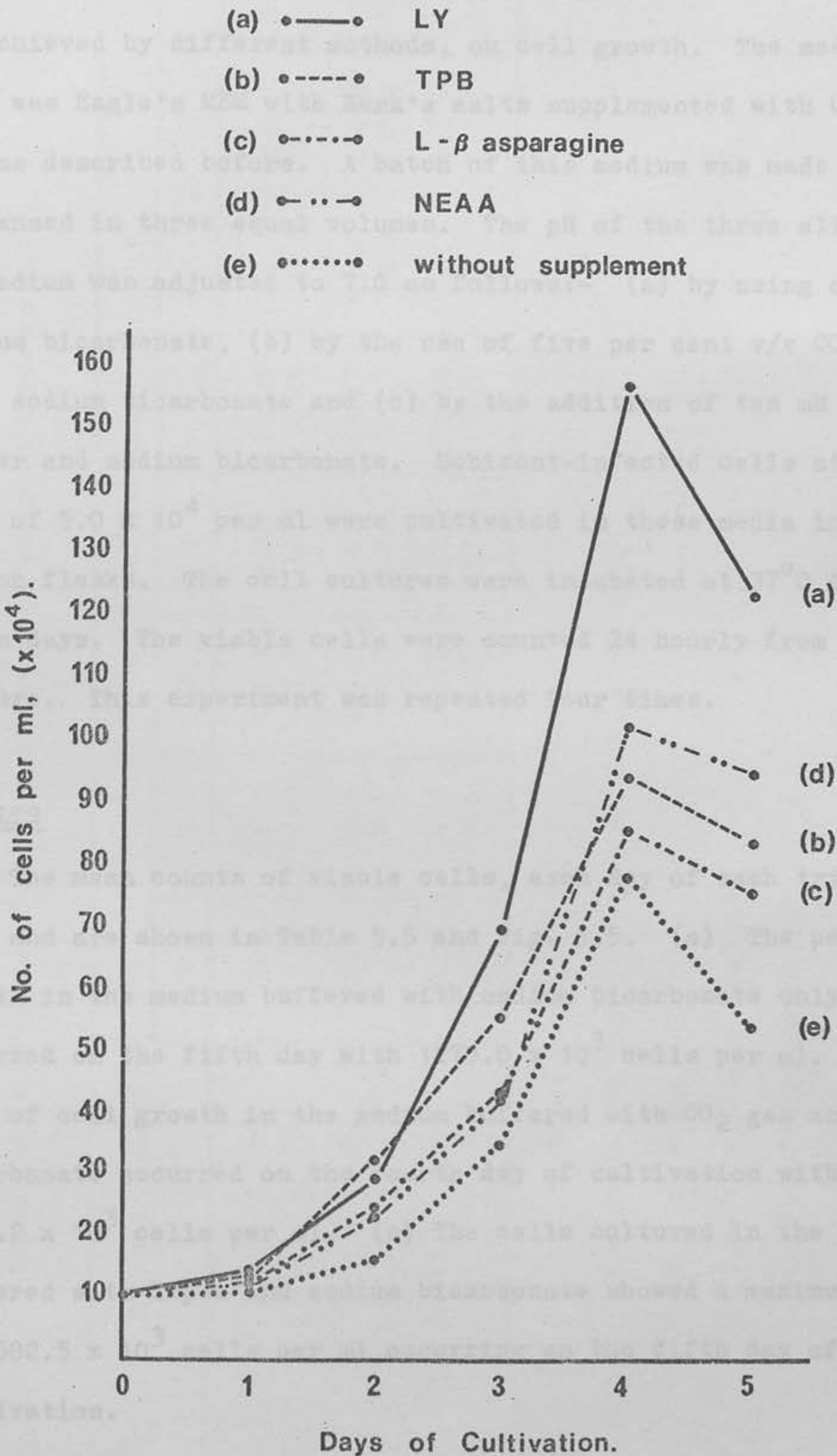
S.D. = Standard Deviation

Experiment 5.5

Effect of pH of the medium on growth rate of schizont-infected cells.

It is generally accepted that for the growth of mammalian cells the optimal pH is between 7.2 and 7.4. In several preliminary

Fig. 5·4 Growth rate of schizont-infected cells cultivated in Eagle's MEM with Hank's salts and various supplements.



trials it was found that schizont-infected cells grew more readily at a pH of approximately 7.0. On the other hand, the kind of buffer appeared to affect the pH and consequently the growth of the cells. This experiment was planned to examine the effect of pH achieved by different methods, on cell growth. The medium used was Eagle's MEM with Hank's salts supplemented with CS and LY, as described before. A batch of this medium was made up and dispensed in three equal volumes. The pH of the three aliquots of medium was adjusted to 7.0 as follows:- (a) by using only sodium bicarbonate, (b) by the use of five per cent v/v $CO_2$ gas with sodium bicarbonate and (c) by the addition of ten mM Hepes buffer and sodium bicarbonate. Schizont-infected cells at the rate of $5.0 \times 10^4$ per ml were cultivated in these media in 75 $cm^2$ Falcon flasks. The cell cultures were incubated at $37^oC$ for seven days. The viable cells were counted 24 hourly from each culture. This experiment was repeated four times.

RESULTS

The mean counts of viable cells, each day of each trial, were made and are shown in Table 5.5 and Fig. 5.5. (a) The peak of growth in the medium buffered with sodium bicarbonate only, occurred on the fifth day with $1275.0 \times 10^3$ cells per ml. (b) The peak of cell growth in the medium buffered with $CO_2$ gas and sodium bicarbonate occurred on the fourth day of cultivation with $1411.2 \times 10^3$ cells per ml. (c) The cells cultured in the medium buffered with Hepes and sodium bicarbonate showed a maximum growth of $1582.5 \times 10^3$ cells per ml occurring on the fifth day of cultivation.

Table 5.5

Effect of pH of the medium on growth rate of schizont-infected cells.

| Buffer | Cells/ ml | Days of cultivation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (a) | M. | 50.0 | 66.2 | 138.7 | 358.7 | 688.7 | 1275.0 | 853.7 | 517.5 |
| | S.D. | | 27.8 | 23.9 | 74.2 | 141.8 | 227.5 | 101.7 | 62.4 |
| (b) | M. | 50.0 | 86.2 | 195.0 | 545.0 | 1411.2 | 1251.2 | 910.0 | 686.2 |
| | S.D. | | 18.9 | 46.9 | 165.6 | 106.6 | 149.4 | 130.4 | 150.1 |
| (c) | M. | 50.0 | 98.7 | 206.2 | 372.5 | 795.0 | 1582.5 | 1451.2 | 1125.0 |
| | S.D. | | 18.0 | 42.5 | 117.4 | 78.2 | 54.8 | 242.0 | 105.1 |

Nos. of cells per ml, x $10^3$

The above are mean cell counts of four cultures.

M. = Mean

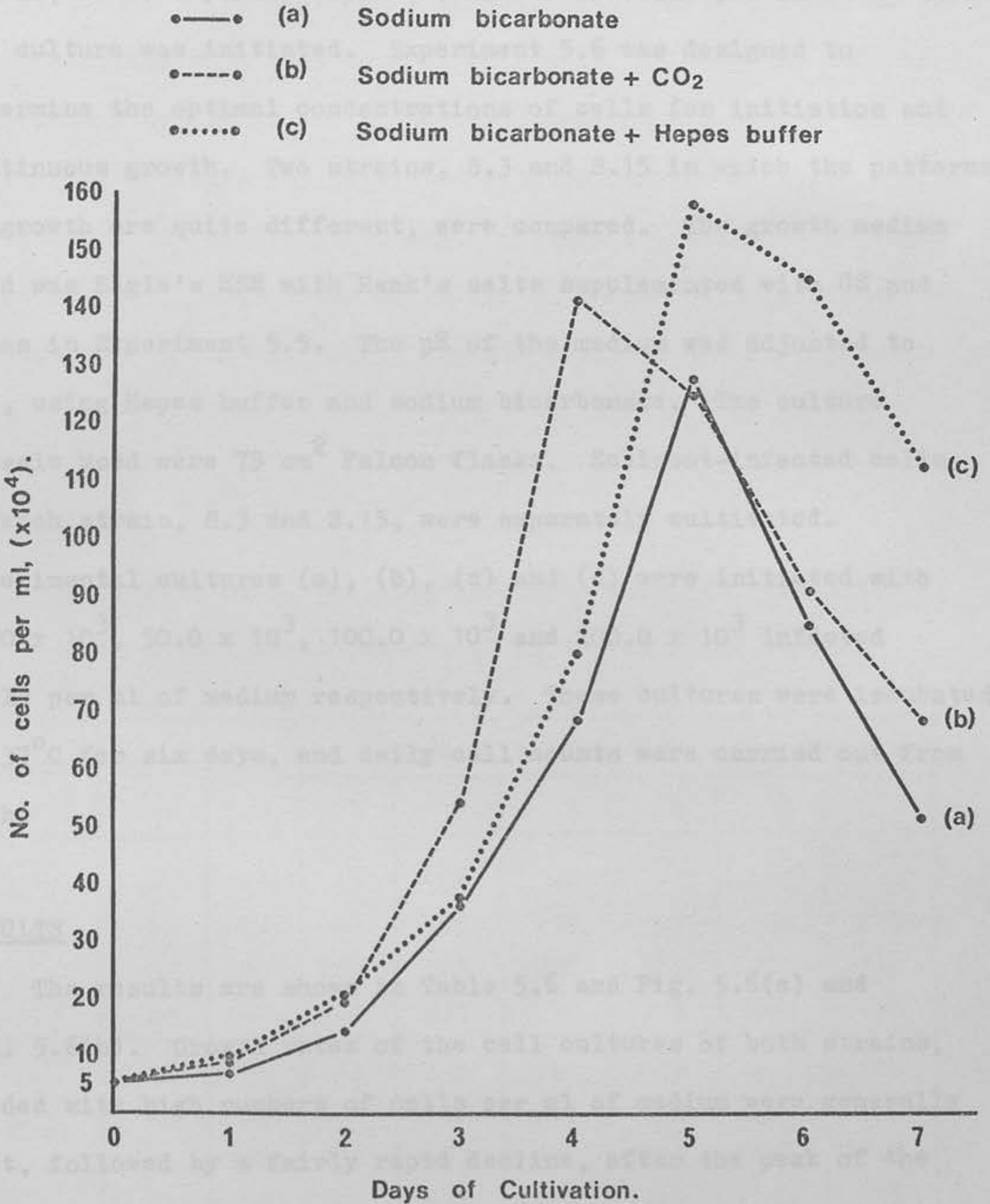
S.D. = Standard Deviation

(a) - Sodium bicarbonate.

(b) - Sodium bicarbonate plus $CO_2$.

(c) - Sodium bicarbonate plus Hepes buffer.

Fig. 5·5 Effect of pH of the medium on growth rate of schizont-infected cells.



Experiment 5.6

Growth rates of T. annulata-infected cells, S.3 and S.15 seeded with various numbers of cells per ml of medium.

In Experiment 5.1, it was demonstrated that different strains of T. annulata-infected cells could have different patterns of growth. This pattern was also observed, in several preliminary trials, to be dependent upon the number of cells per ml with which the culture was initiated. Experiment 5.6 was designed to determine the optimal concentrations of cells for initiation and continuous growth. Two strains, S.3 and S.15 in which the patterns of growth are quite different, were compared. The growth medium used was Eagle's MEM with Hank's salts supplemented with CS and LY as in Experiment 5.5. The pH of the medium was adjusted to 7.0, using Hepes buffer and sodium bicarbonate. The culture vessels used were 75 $cm^2$ Falcon flasks. Schizont-infected cells of each strain, S.3 and S.15, were separately cultivated. Experimental cultures (a), (b), (c) and (d) were initiated with $25.0 \times 10^3$, $50.0 \times 10^3$, $100.0 \times 10^3$ and $200.0 \times 10^3$ infected cells per ml of medium respectively. These cultures were incubated at $37^oC$ for six days, and daily cell counts were carried out from each.

RESULTS

The results are shown in Table 5.6 and Fig. 5.6(a) and Fig. 5.6(b). Growth rates of the cell cultures of both strains, seeded with high numbers of cells per ml of medium were generally fast, followed by a fairly rapid decline, after the peak of the growth. The growth rates were slow in the cultures started with low numbers of seed cells. The growth patterns of schizont-infected cells of S.3 were as follows:- Culture (a) initiated

with 25.0 x $10^3$ cells per ml showed only a slight increase to 65.0 x $10^3$ cells in two days, after which they died off. Culture (b) initiated with 50.0 x $10^3$ cells per ml attained a peak of 490.0 x $10^3$ on the fifth day. Culture (c) initiated with 100.0 x $10^3$ cells and (d) with 200.0 x $10^3$ cells per ml, increased to 980 x $10^3$ cells per ml on the fourth day and 1050 x $10^3$ cells per ml on the third day, respectively. The growth pattern of the cells infected with S.15 were as follows:- Culture (a) initiated with 25.0 x $10^3$ cells per ml showed a peak of 800.0 x $10^3$ on the fifth day. Culture (b) initiated with 50.0 x $10^3$ attained a peak of 1710.0 x $10^3$ on the fifth day. Culture (c) seeded with 100.0 x $10^3$ and (d), 200.0 x $10^3$ cells per ml showed cell increases up to 1825.0 x $10^3$ on the fourth day and 1735.0 x $10^3$ on the third day.

Table 5.6

Growth rate of T. annulata-infected cells, S.3 and S.15 seeded with various numbers of cells per ml of medium.

| Strain | Trial | Days of cultivation | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| S.3 | a | 25 | 30 | 65 | 20 | 5 | 0 | 0 |
| | b | 50 | 60 | 125 | 290 | 405 | 490 | 310 |
| | c | 100 | 180 | 425 | 750 | 980 | 810 | 440 |
| | d | 200 | 340 | 815 | 1050 | 725 | 600 | 230 |
| S.15 | a | 25 | 40 | 115 | 150 | 635 | 800 | 750 |
| | b | 50 | 90 | 220 | 560 | 1330 | 1710 | 1500 |
| | c | 100 | 205 | 480 | 950 | 1825 | 1515 | 1050 |
| | d | 200 | 460 | 1075 | 1735 | 1410 | 1150 | 375 |

No. of cells per ml, x $10^3$

The figures are means of duplicate cell counts of two cultures.

Fig. 5·6 (a) Growth rate of T. annulata - infected cells, S.3, seeded with various numbers of cells per ml medium.

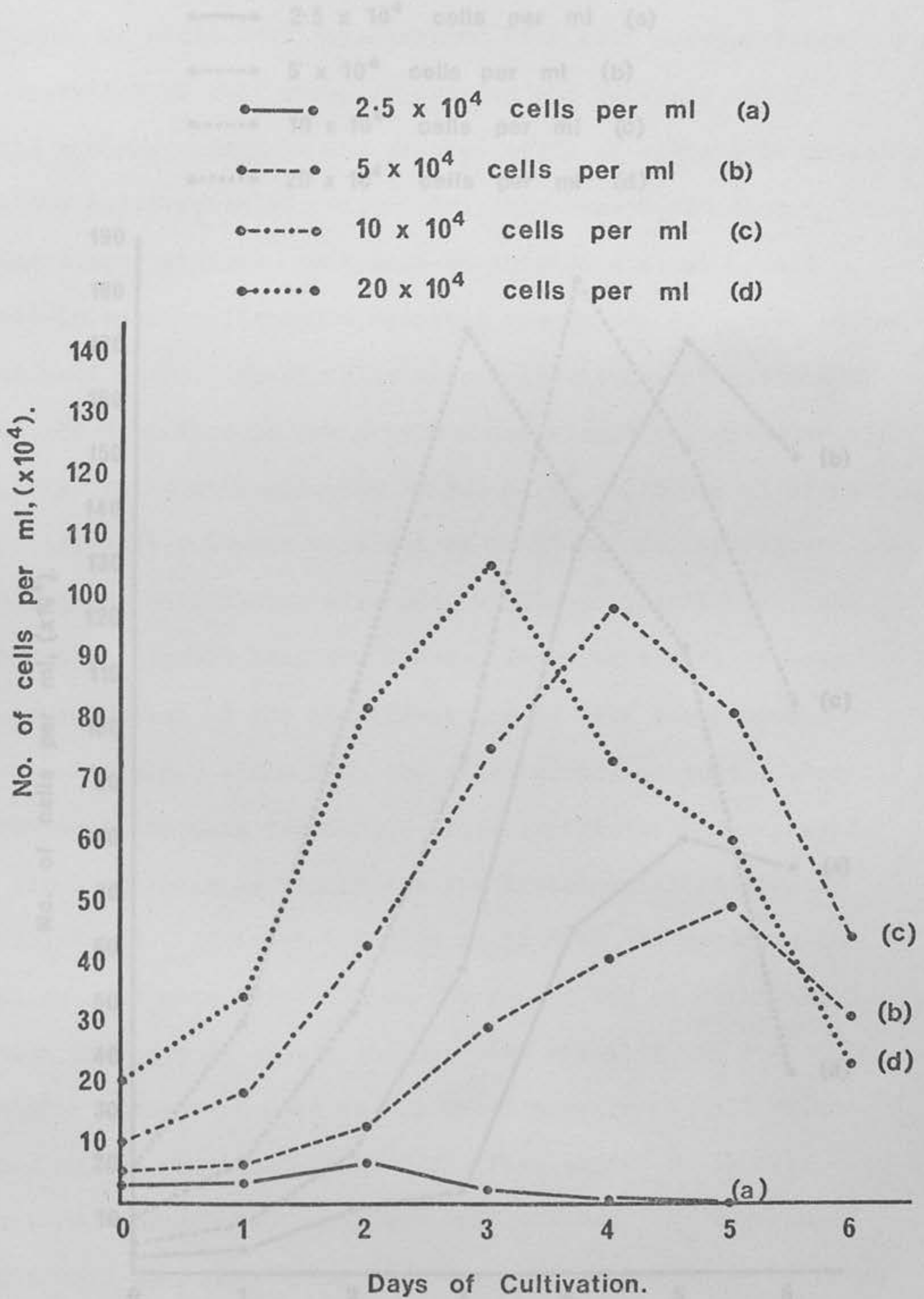
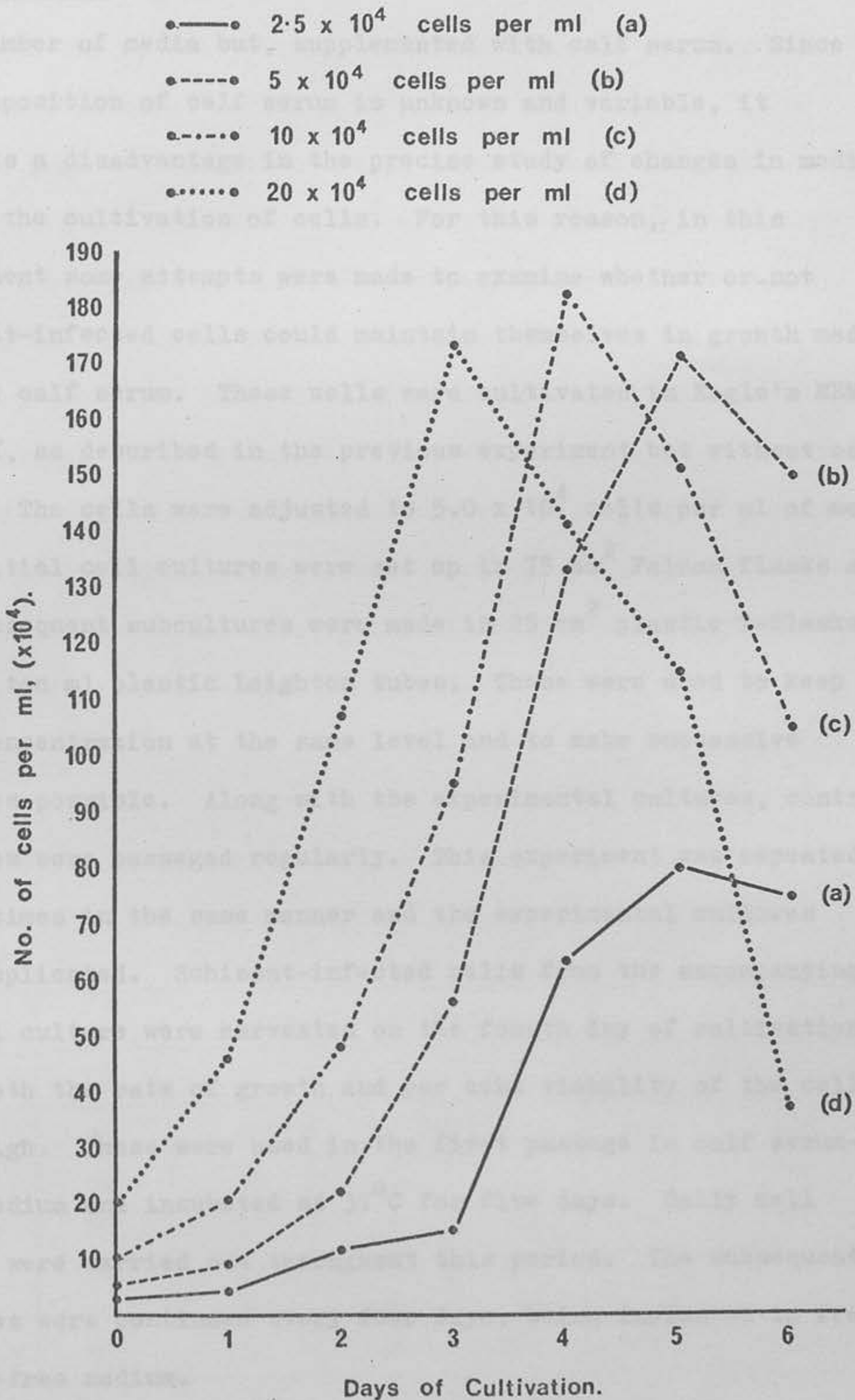


Fig. 5·6 (b) Growth rate of T. annulata- infected cells, S.15, seeded with various numbers of cells per ml medium.



Experiment 5.7

Attempt to grow schizont-infected cells without calf serum.

*Theileria*-infected cells have been successfully cultivated in a number of media but, supplemented with calf serum. Since the composition of calf serum is unknown and variable, it presents a disadvantage in the precise study of changes in media during the cultivation of cells. For this reason, in this experiment some attempts were made to examine whether or not schizont-infected cells could maintain themselves in growth medium without calf serum. These cells were cultivated in Eagle's MEM with LY, as described in the previous experiment but without calf serum. The cells were adjusted to $5.0 \times 10^4$ cells per ml of medium. The initial cell cultures were set up in 75 $cm^2$ Falcon flasks and the subsequent subcultures were made in 25 $cm^2$ plastic T-flasks and in ten ml plastic Leighton tubes. These were used to keep cell concentration at the same level and to make successive passages possible. Along with the experimental cultures, control cultures were passaged regularly. This experiment was repeated three times in the same manner and the experimental cultures were duplicated. Schizont-infected cells from the accompanying control culture were harvested on the fourth day of cultivation when both the rate of growth and per cent viability of the cells were high. These were used in the first passage in calf serum-free medium and incubated at 37°C for five days. Daily cell counts were carried out throughout this period. The subsequent passages were continued every four days, being implanted in fresh but CS-free medium.

RESULTS

The results are shown in Table 5.7 and Fig. 5.7. The rates of growth in the first passages of all trials were high. Onset of cell growth represented 1200.0 x $10^3$ cells per ml of medium in the fourth day of cultivation, although this was less than that of the control culture, 1815.0 x $10^3$.

The peak numbers of the cells in the second and third passages were 720.0 x $10^3$ and 400.0 x $10^3$ cells per ml of medium, respectively. Both of these peaks occurred on the fourth day of cultivation. Finally, in the fourth passage, schizont-infected cells increased to only 210.0 x $10^3$ three days after cultivation, and in the fifth passage the cells merely survived for three days then they died off. Cell numbers diminished in every successive passage, so that in the fifth passage there were not enough viable cells to maintain the cultures. Repetition of the experiment on two further occasions gave similar results.

Table 5.7

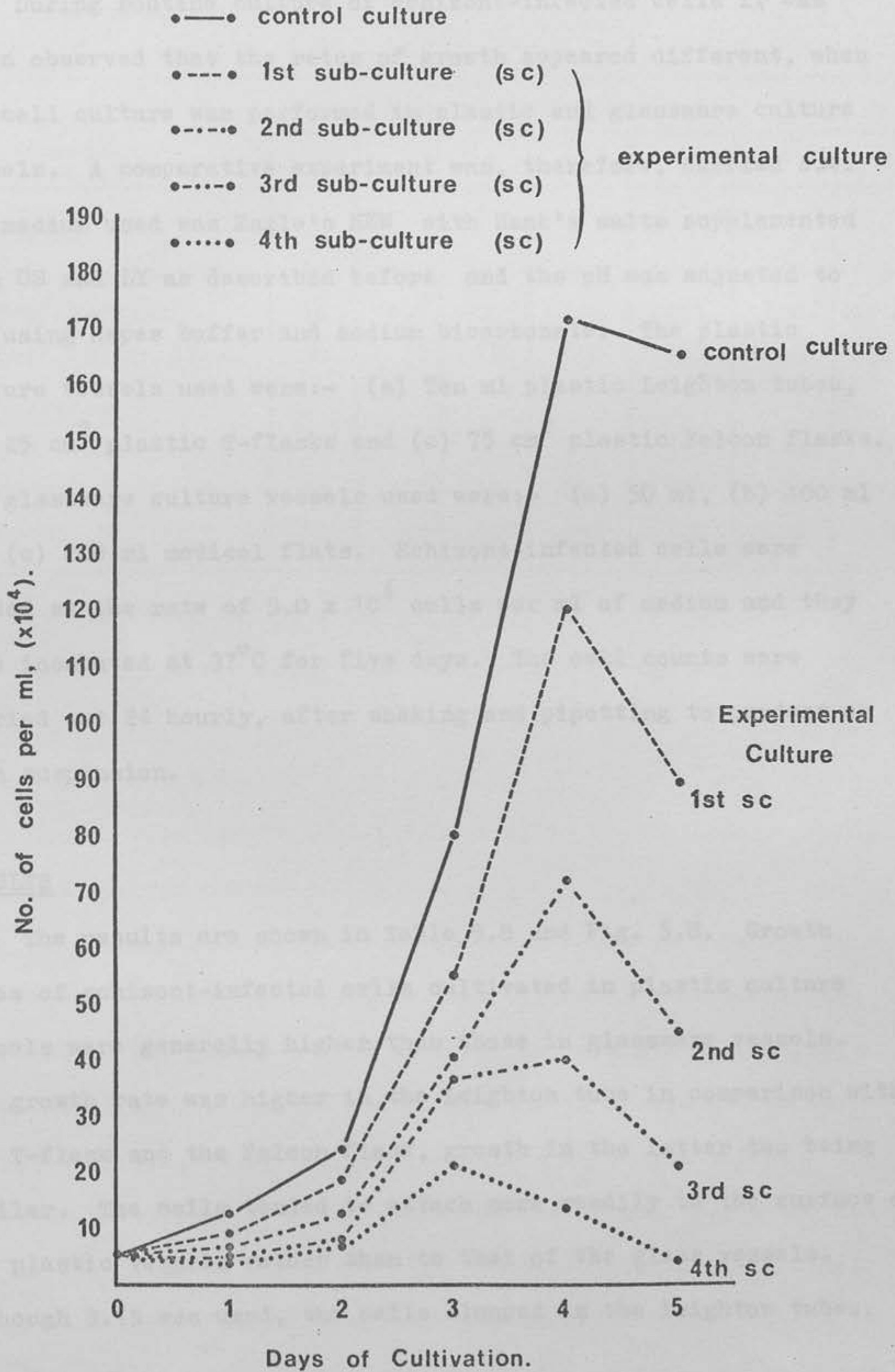
Growth rate of schizont-infected cells in Eagle's MEM with LY and without CS.

| Passage | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| 1 | 50 | 90 | 185 | 550 | 1200 | 895 |
| 2 | 50 | 65 | 125 | 405 | 720 | 450 |
| 3 | 50 | 50 | 80 | 365 | 400 | 210 |
| 4 | 50 | 35 | 65 | 210 | 135 | 40 |
| 5 | 50 | 30 | 25 | 10 | 0 | 0 |
| Control | 50 | 125 | 240 | 800 | 1710 | 1650 |

No. of cells per ml, x $10^3$

The figures are means of duplicate cell counts from five successive passages.

Fig. 5·7 Growth rate of schizont-infected cells in Eagle's MEM with LY and without CS.



Experiment 5.8

Growth rate of schizont-infected cells in plastic and glassware culture vessels.

During routine culture of schizont-infected cells it was often observed that the rates of growth appeared different, when the cell culture was performed in plastic and glassware culture vessels. A comparative experiment was, therefore, carried out. The medium used was Eagle's MEM with Hank's salts supplemented with CS and LY as described before and the pH was adjusted to 7.0 using Hepes buffer and sodium bicarbonate. The plastic culture vessels used were:- (a) Ten ml plastic Leighton tubes, (b) 25 $cm^2$ plastic T-flasks and (c) 75 $cm^2$ plastic Falcon flasks. The glassware culture vessels used were:- (a) 50 ml, (b) 100 ml and (c) 200 ml medical flats. Schizont-infected cells were seeded at the rate of $5.0 \times 10^4$ cells per ml of medium and they were incubated at 37°C for five days. The cell counts were carried out 24 hourly, after shaking and pipetting to produce an even suspension.

RESULTS

The results are shown in Table 5.8 and Fig. 5.8. Growth rates of schizont-infected cells cultivated in plastic culture vessels were generally higher than those in glassware vessels. The growth rate was higher in the Leighton tube in comparison with the T-flask and the Falcon flask, growth in the latter two being similar. The cells tended to attach more readily to the surface of the plastic vessels rather than to that of the glass vessels. Although S.15 was used, the cells clumped in the Leighton tubes.

Table 5.8

A comparison between growth rate of schizont-infected cells cultivated in plastic culture vessels and in glass medical flats.

| Culture vessels | Days of cultivation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Plastic | | | | | | | |
| 10ml Leighton tubes | 50 | 140 | 375 | 790 | 1800 | 1265 | 890 |
| $25cm^2$ T-flask | 50 | 125 | 305 | 590 | 1425 | 1690 | 1110 |
| $75cm^2$ Falcon flask | 50 | 95 | 220 | 550 | 1250 | 1615 | 1325 |
| Glassware | | | | | | | |
| 50ml medical Flat | 50 | 105 | 300 | 585 | 1330 | 1390 | 905 |
| 100 ml " " | 50 | 125 | 315 | 620 | 1175 | 1445 | 1260 |
| 200 ml " " | 50 | 85 | 255 | 490 | 1320 | 1280 | 960 |

No. of cells per ml x $10^3$

The figures represent mean cell counts of two cultures.

Experiment 5.9

Growth rate of schizont-infected cells in stationary and stirred cultures.

To examine whether schizont-infected cells grow better in stationary or in stirred culture, the following experiment was performed. The growth medium used was Eagle's MEM with Earle's salts supplemented with CS and LY and the pH of the medium was adjusted to 7.0, using sodium bicarbonate and Hepes buffer. Schizont-infected cells were seeded at the rate of 5.0 x $10^4$ cells per ml of medium as follows:- (a) In two 200 ml medical flats which were kept stationary without any magnetic bar. (b) In two 200 ml medical flats, each containing a two cm long silicone-coated

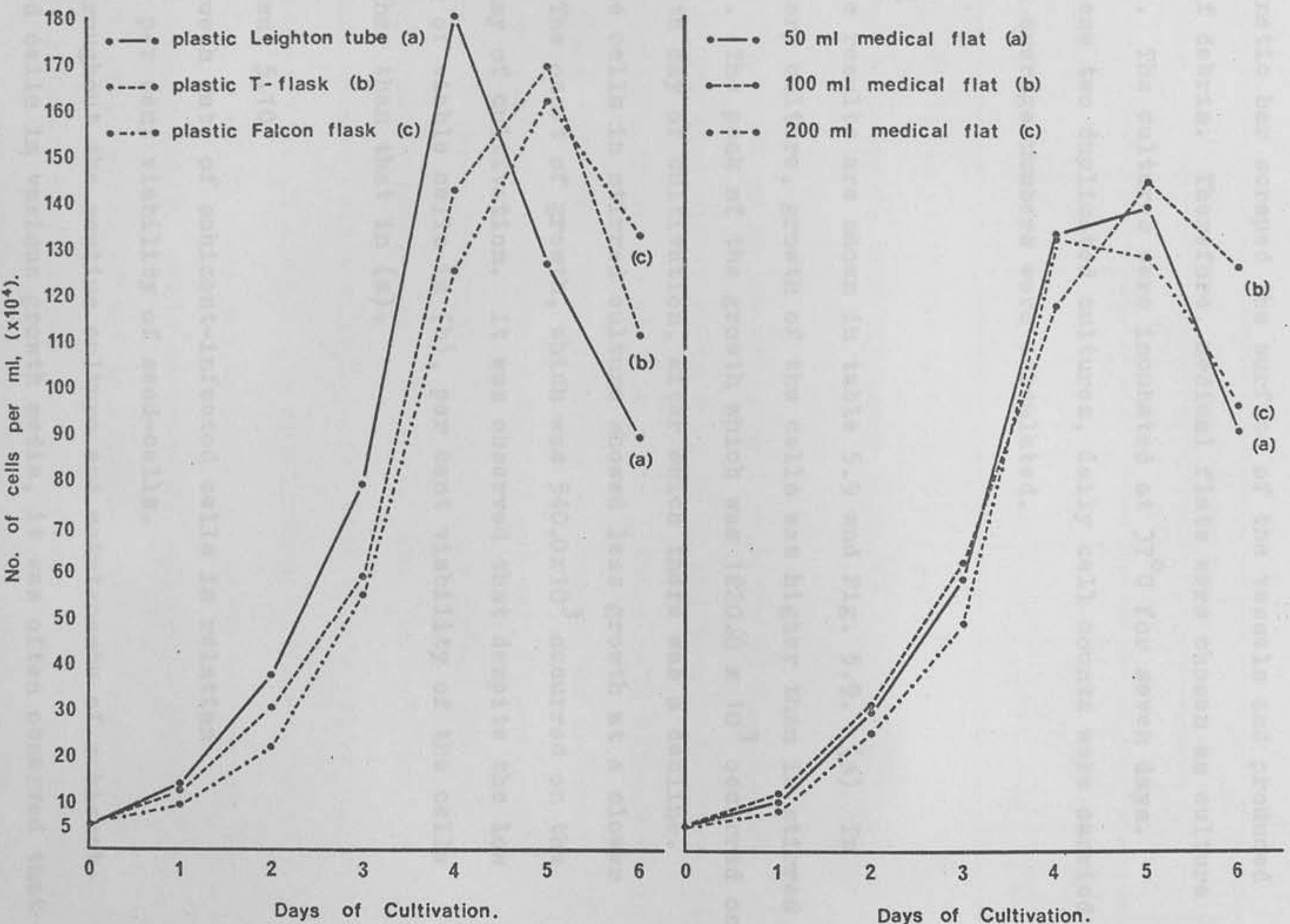



Fig. 5·8 A comparison between growth rate of schizont-infected cells cultivated in plastic culture vessels and in glass medical flats.

magnetic bar. These were set up on an electric magnetic stirrer. The magnetic bar rotated at 250 rpm. This experiment was carried out first using plastic culture vessels but, it was observed that the magnetic bar scraped the surface of the vessels and produced a lot of debris. Therefore, medical flats were chosen as culture vessels. The cultures were incubated at 37°C for seven days. From these two duplicated cultures, daily cell counts were carried out and average numbers were calculated.

RESULTS

The results are shown in table 5.9 and Fig. 5.9. (a) In stationary culture, growth of the cells was higher than in stirred culture. The peak of the growth which was $1220.0 \times 10^3$ occurred on the fifth day of cultivation, after which there was a decline. (b) The cells in stirred culture showed less growth at a slower rate. The onset of growth, which was $540.0 \times 10^3$ occurred on the sixth day of cultivation. It was observed that despite the low numbers of viable cells in (b), per cent viability of the cells was higher than that in (a).

Experiment 5.10

Growth rate of schizont-infected cells in relation to per cent viability of seed-cells.

Throughout the routine culture and maintenance of schizont-infected cells in various growth media, it was often observed that the rates of growth were variable. It was thought that this could be due to per cent viability (% V) of the cells which varied from one culture to another. It should be mentioned here that the cells used in the initial cultures in these experiments were harvested

Table 5.9

Growth rates of schizont-infected cells cultivated in stationary and stirred cultures.

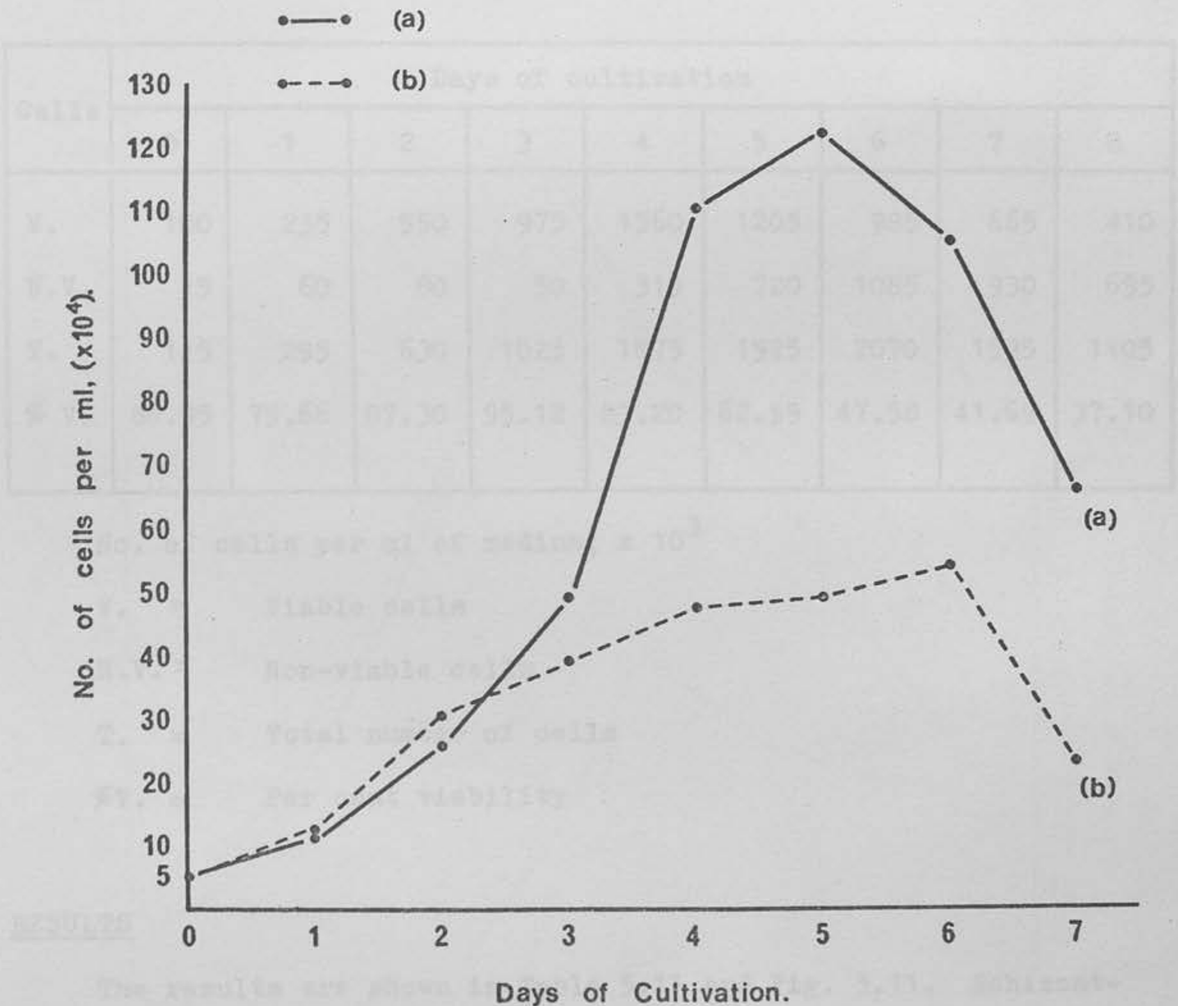
| Culture | Days of cultivation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Stationary | 50 | 110 | 255 | 490 | 1100 | 1220 | 1050 | 660 |
| Stirred | 50 | 125 | 305 | 390 | 475 | 490 | 540 | 230 |

No. of viable cells per ml of medium, x $10^3$

Mean cell counts from duplicate cultures.

from active cultures in the third or fourth day of the passage, with high per cent viability from 65 to 75 and more. To determine to what extent, if any, viability of an initial cell culture could affect the growth rate, the following experiment was designed. Several preliminary trials were carried out and, finally, cell cultures with three levels of viability were chosen for the experiment, as follows:- (a) 95.12%V. (b) 62.59%V. (c) 41.69%V. The growth medium used was Eagle's MEM with Hank's salts supplemented with CS and LY. To obtain an even suspension of seed-cells, a culture was set up using the above medium, in a plastic Falcon flask. Schizont-infected cells were adjusted to 1.0 x $10^5$ cells per ml of medium and incubated for eight days. Daily cell counts were carried out and viable (V.) and non-viable (N.V.) cells were counted. Cells from this culture in the third, fifth and seventh day of cultivation were used for this experiment. Also from this stock culture, the pattern of growth was as described in Table 5.10 and Fig. 5.10. The numbers of

Fig. 5·9 Growth rate of schizont-infected cells (a) in stationary culture and (b) in stirred culture.



schizont-infected cells were then adjusted to $5.0 \times 10^4$ cells per ml of medium in all cultures. These were set up in 25 $cm^2$ T-flasks and incubated at $37^oC$ for six days. Cell counts were carried out from samples taken from the cultures at 24 hourly intervals.

Table 5.10

Growth rate of schizont-infected cells used as culture in Experiment 5.10

| Cells | Days of cultivation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| V. | 100 | 235 | 550 | 975 | 1560 | 1205 | 985 | 665 | 410 |
| N.V. | 15 | 60 | 80 | 50 | 315 | 720 | 1085 | 930 | 695 |
| T. | 115 | 295 | 630 | 1025 | 1875 | 1925 | 2070 | 1595 | 1105 |
| % V. | 86.95 | 79.66 | 87.30 | 95.12 | 83.20 | 62.59 | 47.58 | 41.69 | 37.10 |

No. of cells per ml of medium, $\times 10^3$

V. = Viable cells

N.V. = Non-viable cells

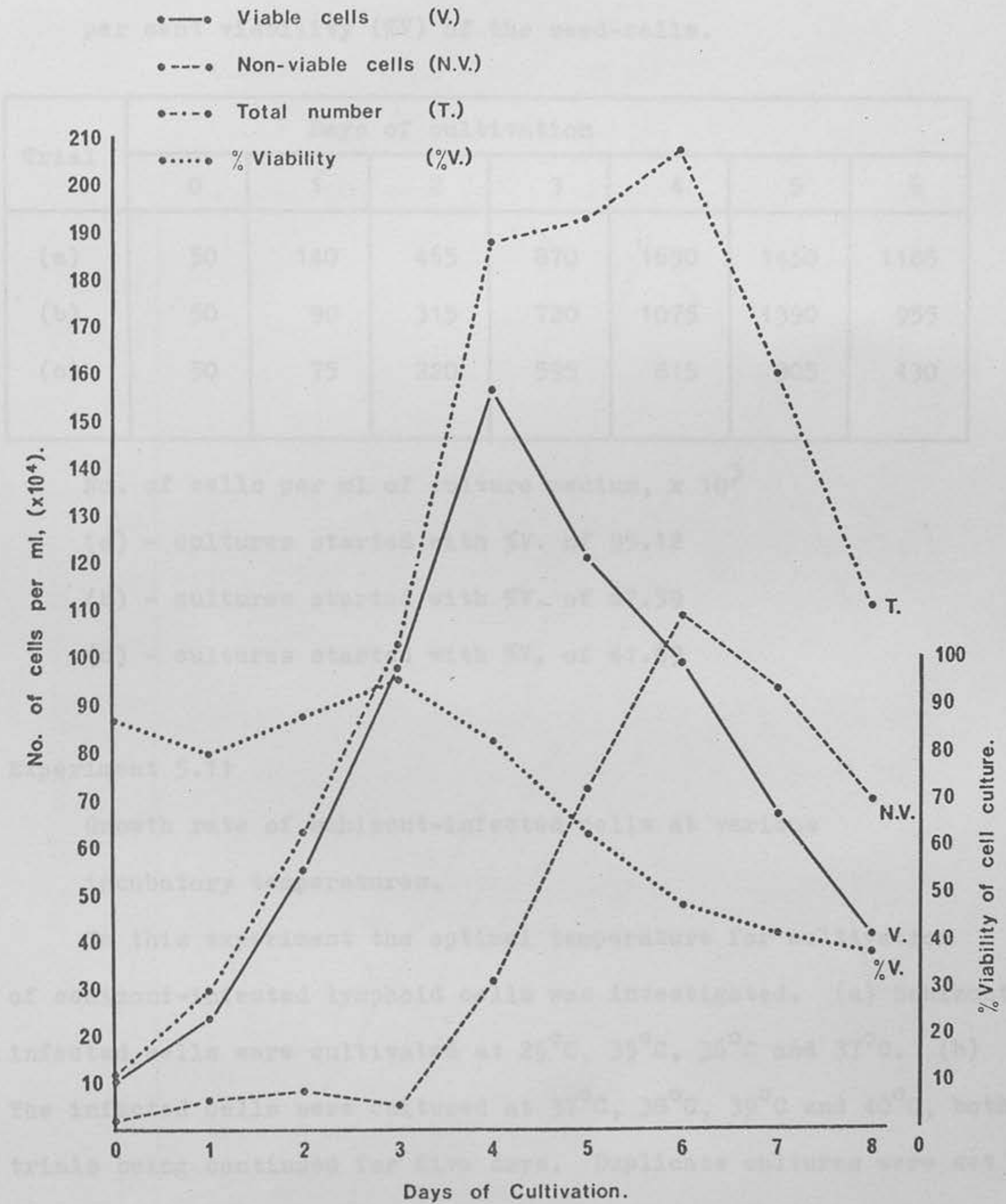
T. = Total number of cells

%V. = Per cent viability

RESULTS

The results are shown in Table 5.11 and Fig. 5.11. Schizont-infected cells in trial (a) showed a high growth with the peak occurring on the fourth day. (b) These cells presented a lower growth rate than the previous one, with the peak of growth occurring on the fifth day. (c) The cells with low %V. showed a poor growth compared with those of the two others, (a) and (b).

Fig. 5·10 Pattern of growth of schizont-infected cells cultivated in Eagle's MEM with Hank's salts and supplemented with CS and LY.



The peak of growth occurred on the fifth day of cultivation.

Table 5.11

Growth rate of schizont-infected cells in relation to per cent viability (%V) of the seed-cells.

| Trial | Days of cultivation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| (a) | 50 | 140 | 465 | 870 | 1690 | 1450 | 1185 |
| (b) | 50 | 90 | 315 | 720 | 1075 | 1390 | 955 |
| (c) | 50 | 75 | 220 | 595 | 815 | 905 | 430 |

No. of cells per ml of culture medium, x $10^3$

(a) - cultures started with %V. of 95.12

(b) - cultures started with %V. of 62.59

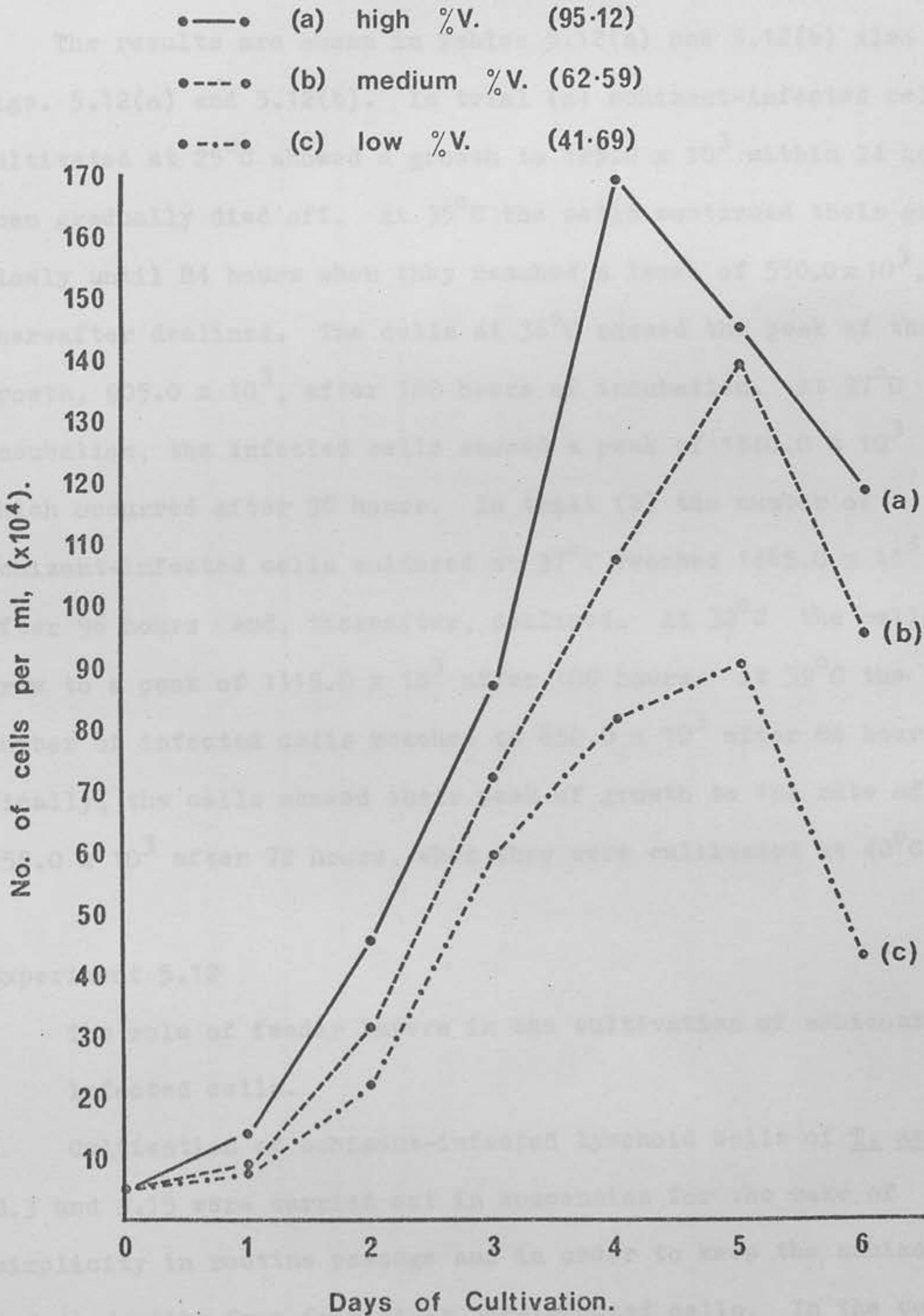
(c) - cultures started with %V. of 41.69

Experiment 5.11

Growth rate of schizont-infected cells at various incubatory temperatures.

In this experiment the optimal temperature for cultivation of schizont-infected lymphoid cells was investigated. (a) Schizont-infected cells were cultivated at 25°C, 35°C, 36°C and 37°C. (b) The infected cells were cultured at 37°C, 38°C, 39°C and 40°C, both trials being continued for five days. Duplicate cultures were set up at each temperature. The growth medium used was Eagle's MEM with Hank's salts and supplemented with CS and LY. The culture vessels used were 25 $cm^2$ T-flasks. The pH of the medium was adjusted to 7.0, using sodium bicarbonate and Hepes buffer. The

Fig. 5·11 Growth rate of schizont-infected cells in relation to %V. of the seed-cells.



cells were adjusted to $5.0 \times 10^4$ per ml of medium in all cultures. Growth rates of the cells were measured at twelve hourly intervals, from the duplicate cultures.

RESULTS

The results are shown in Tables 5.12(a) and 5.12(b) also in Figs. 5.12(a) and 5.12(b). In trial (a) schizont-infected cells cultivated at $25^{\circ}C$ showed a growth to $125.0 \times 10^3$ within 24 hours, then gradually died off. At $35^{\circ}C$ the cells continued their growth slowly until 84 hours when they reached a level of $550.0 \times 10^3$, and thereafter declined. The cells at $36^{\circ}C$ showed the peak of their growth, $905.0 \times 10^3$, after 108 hours of incubation. At $37^{\circ}C$ incubation, the infected cells showed a peak of $1540.0 \times 10^3$ which occurred after 96 hours. In trial (b) the number of schizont-infected cells cultured at $37^{\circ}C$ reached $1465.0 \times 10^3$ after 96 hours and, thereafter, declined. At $38^{\circ}C$ the cells grew to a peak of $1115.0 \times 10^3$ after 108 hours. At $39^{\circ}C$ the number of infected cells reached to $650.0 \times 10^3$ after 84 hours. Finally, the cells showed their peak of growth to the rate of $255.0 \times 10^3$ after 72 hours, when they were cultivated at $40^{\circ}C$.

Experiment 5.12

The role of feeder layers in the cultivation of schizont-infected cells.

Cultivation of schizont-infected lymphoid cells of T. annulata, S.3 and S.15 were carried out in suspension for the sake of simplicity in routine passage and in order to keep the schizont-infected cells free from other non-infected cells. In the course of routine maintenance of S.3, the infected cells underwent a

Table 5.12(a)

Growth rates of schizont-infected cells at various temperatures

| Incubation temperature (°C) | Hours of cultivation | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 | 108 | 120 |
| 25 | 50 | 45 | 125 | 85 | 60 | 50 | 45 | 50 | 35 | 20 | 5 |
| 35 | 50 | 55 | 95 | 150 | 200 | 310 | 375 | 550 | 305 | 120 | 55 |
| 36 | 50 | 65 | 105 | 160 | 305 | 500 | 555 | 660 | 735 | 905 | 890 |
| 37 | 50 | 65 | 115 | 180 | 395 | 695 | 805 | 1285 | 1540 | 1200 | 1135 |

No. of viable cells per ml of medium, x $10^3$

The above values represent duplicate counts from two cultures.

Fig. 5·12, Trial (a). A Comparison between growth rates of schizont-infected cells at different incubatory temperatures.

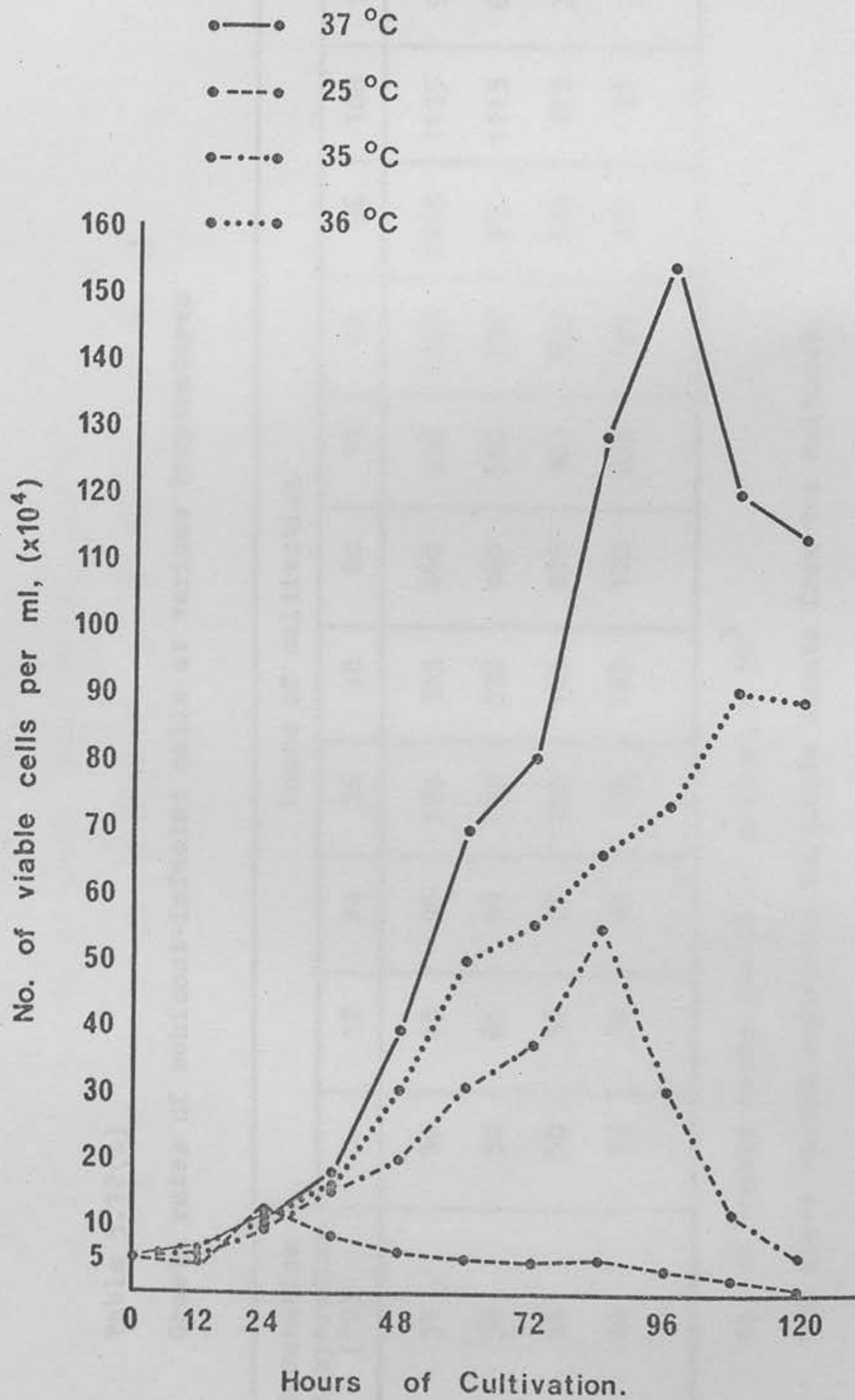


Table 5.12(b)

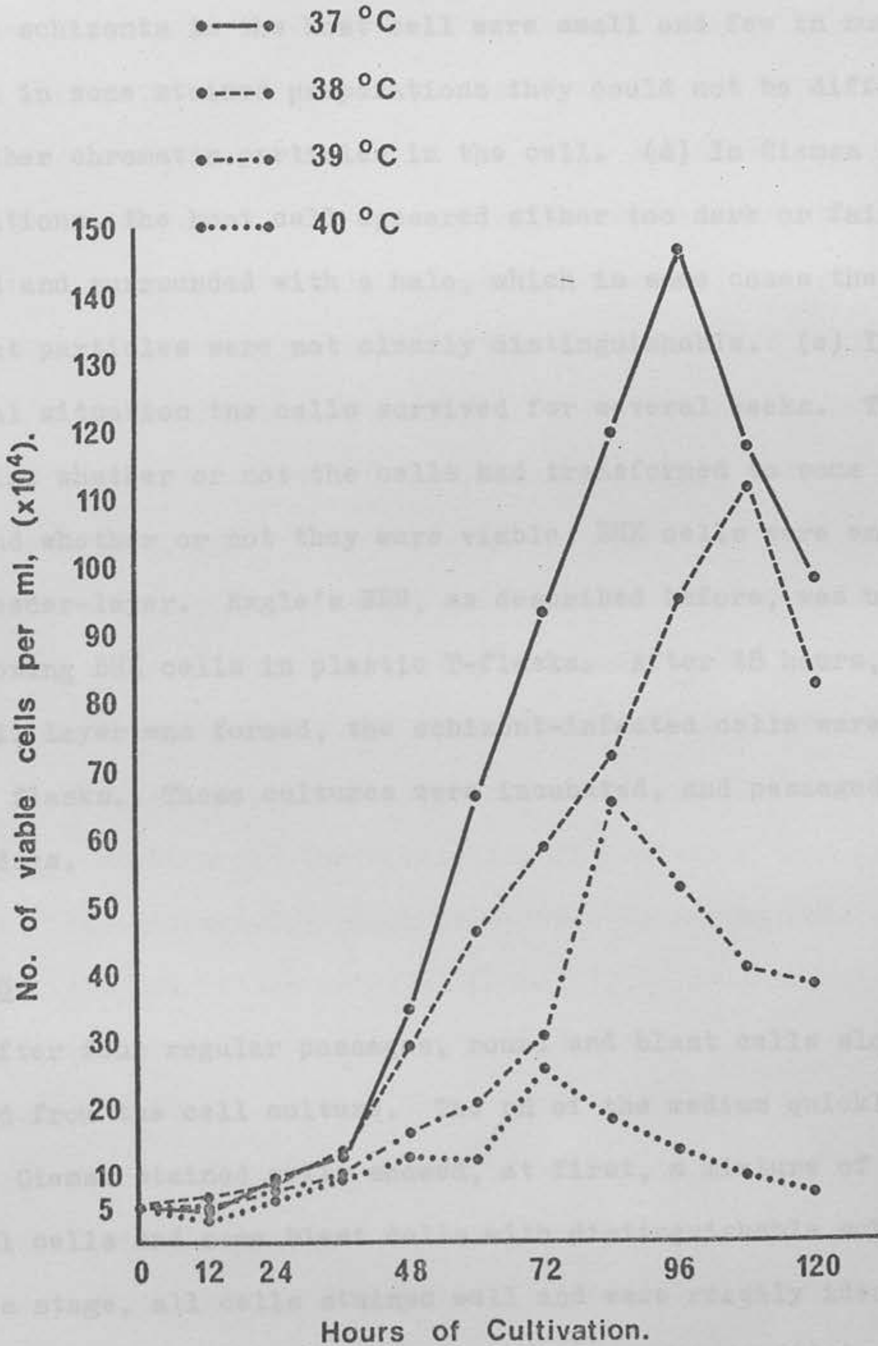
Growth rates of schizont-infected cells at various temperatures

| Incubation temperature (°C) | Hours of cultivation | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 | 108 | 120 |
| 37 | 50 | 45 | 95 | 125 | 345 | 660 | 930 | 1195 | 1465 | 1175 | 980 |
| 38 | 50 | 65 | 90 | 135 | 290 | 460 | 585 | 720 | 950 | 1115 | 825 |
| 39 | 50 | 50 | 75 | 100 | 160 | 205 | 305 | 650 | 525 | 405 | 380 |
| 40 | 50 | 30 | 60 | 90 | 125 | 120 | 255 | 180 | 135 | 95 | 70 |

No. of viable cells per ml of medium, x $10^3$

The above values represent duplicate counts from two cultures.

Fig. 5·12, Trial (b). A Comparison between growth rates of schizont-infected cells at various incubatory temperatures.



change in growth character. (a) The increase in growth which usually varies from 5.35 fold, (Experiment 5.1) to 9.8 fold, (Experiment 5.6) in four days, slowed down considerably within this period. (b) The schizont-infected cells did not exclude trypan blue stain although multiplication of cells occurred. (c) The schizonts in the host cell were small and few in number, so that in some stained preparations they could not be differentiated from other chromatin particles in the cell. (d) In Giemsa stained preparations, the host cell appeared either too dark or faintly stained and surrounded with a halo, which in some cases the schizont particles were not clearly distinguishable. (e) In this abnormal situation the cells survived for several weeks. To determine whether or not the cells had transformed to some other kind and whether or not they were viable, BHK cells were employed as a feeder-layer. Eagle's MEM, as described before, was used for growing BHK cells in plastic T-flasks. After 48 hours, when the cell layer was formed, the schizont-infected cells were added to the flasks. These cultures were incubated, and passaged every three days.

RESULTS

After four regular passages, round and blast cells slowly emerged from the cell culture. The pH of the medium quickly became acid. Giemsa stained cells showed, at first, a mixture of the unusual cells and some blast cells with distinguishable schizonts. At this stage, all cells stained well and were readily identifiable but many did not contain schizonts. These were assumed to be BHK cells. Successive sub-cultures were made using only the suspended cells with the result that in these sub-cultures the

proportion of schizont-infected cells increased.

DISCUSSION

In Experiment 5.1, considering the fact that the growth medium and all other conditions of cultivation were the same, it was shown that there were differences in rate of growth of the strains used. The high growth rate of S.15 accords with known characteristics of the strain and its ready adaptation *in vitro*. It has been observed by the present author to show an ability to adhere to the surface of culture vessels, where it rapidly establishes itself and grows fast, Fig. 5.13. It does not tend to clump as much as does S.3, Fig. 5.14. Both strains produce only the intra-lymphocytic form of the parasite *in vivo* and are well established *in vitro*.

S.15 shows a considerably higher growth rate compared with S.3. This might well be due to the virulence of S.3, which is much higher than that of S.15. Hooshmand-Rad and Hashemi-Fesharki (1968) have shown a positive correlation between the virulence of *T. annulata* strains and the difficulty in isolating them in tissue culture. The differences which were observed in the rate of growth of lymphoid cells infected with S.19, S.20 and S.21 can possibly, therefore, be explained as being due to their varying virulence.

In Experiment 5.2, the growth of schizont-infected cells of S.15 cultivated in the control medium, modified Eagle (1955) supplemented with CS and LY, is of a pattern well established by the experience of several workers (Hooshmand-Rad 1973 and 1975; and Hashemi-Fesharki and Shad-Del 1973a and 1973b). In this medium, the strain multiplied nine or ten fold. The poor growth

of infected cells in the media (a), (b) and (c) can be explained as due to an insufficiency of amino-acids and vitamins which were not included in them, except in so far as they were available in CS and LY. This agrees with the statement of Eagle (1955 and 1959) that certain amino-acids, especially glutamine, and vitamins are essential for growth of the cells whether they are from man or animal and whether they are normal or malignant. Significant differences between the growth rates of the cells in the experimental media in the first and third subcultures and at the first and fourth days of subculture are most probably due to fast exhaustion of the growth factors in the media. Increasing numbers of non-viable cells might also play a role in production of unfavourable conditions, as is indicated later in Experiment 5.10. Slight differences of growth rates of the cells cultivated in the media (a), (b) and (c) might be due to different numbers of salts in (a) compared with (b) and (c).

In Experiment 5.3, the rate of growth in modified Eagle's medium was in the normal range of approximately ten fold. This agrees with the observations reported by Hooshmand-Rad (1973 and 1975). The lower, 9.46 fold growth which was observed using TC medium 199 can be explained as due to a deficiency of required constituents for schizont-infected cells; e.g. glutamine and other amino-acids. High growth rates of the infected cells in Eagle's MEM, either with Hank's salts or Earle's salts and both supplemented with CS and LY, indicate that these are good growth media for these cells. The average rate of decline after the peak of growth shows that, generally, the higher the peak, the more marked the decline. This is explained as to the increased number of non-viable cells in the culture.

In Experiment 5.4, it was observed that the rate of growth was higher when the growth medium contained supplements. The beneficial effects of various supplements on the growth of Theileria-infected cells have been reported by many authors (Hulliger et al 1964; Hulliger 1965; Malmquist et al 1970 and 1974; Hooshmand-Rad 1973 and 1975; Hashemi-Fesharki and Shad-Del 1973a and 1973b; Stagg et al 1974; Danskin and Wilde 1976a and 1976b). The comparison made with some supplements and promotion of growth rates indicates that while the addition of TPB, L -$\beta$ asparagine and NEAA improve the growth, this improvement was much greater when LY was added to the medium. This suggests that for lymphoid cells infected with T. annulata, LY plays an important part in the promotion of growth, presumably as a function of the growth factors which it contains.

Experiment 5.5 produced three patterns of cell increase and decline:- In trial (a) when the pH of the medium was adjusted only by sodium bicarbonate, there was slow growth in the first 48 hours. This period of slow growth is required to bring the cell concentration up to the optimum for initial growth (50.0 x $10^3$ per ml vide. Experiment 5.6). Using sodium bicarbonate alone, the pH of the medium at 37°C initially increases (Leibovitz 1963 and Good et al 1966). Subsequent metabolic activity of the cells brings about a reduction of pH. This is possibly the reason why there is an actual reduction of cells in the period twelve to 24 hours of cultivation. In trial (b) the rapid increase of the cells can be explained as the result of the combination of sodium bicarbonate and five per cent $CO_2$ gas. This combination prevents marked fluctuation of pH from approximately 7.0. In trial (c) the medium which was buffered with sodium

bicarbonate and Hepes provided the cells with a more stable pH. The growth was higher and the decline slower because of the slower change of pH of the medium. Schizont-infected cells grow more readily and faster in a medium of pH 7.0 than with pH 7.2 and higher. This has been confirmed by Hooshmand-Rad in a personal communication (1975). The slow change of pH in a medium buffered with Hepes provides a fairly long and steady cell growth. This is a satisfactory and time-saving means for routine maintenance of these infected cells.

The effect of seed-cell numbers on growth rates of the infected cells is investigated in Experiment 5.6. It is shown that with both strains, S.3 and S.15, the rate and pattern of growth are governed to some extent by the seeding rate of the initial culture. Thus, with S.3 at a seeding level of $2.5 \times 10^4$ per ml, the cells did not become established. Establishment and good growth occurred when the seed rate was $5.0 \times 10^4$ per ml but with seed rates of $10.0 \times 10^4$ per ml and $20.0 \times 10^4$ per ml rapid establishment and good growth were achieved. The peaks attained with the latter two cultures were similar but growth and decline were more rapid with the higher seeding rate. It was, therefore, concluded for routine maintenance that a seed rate of $10.0 \times 10^4$ was optimal having in mind convenience in time and economy of materials. A similar picture is shown with S.15 but in this case optimal growth is obtained with an initial seed level of $5.0 \times 10^4$. It is interesting to note, also, that with this less virulent strain, establishment and growth were obtained with a seed level as low as $2.5 \times 10^4$ per ml.

In Experiment 5.7, attempts were made to grow schizont-infected cells without calf serum in four and five successive

passages. The rate of growth in the first sub-culture was reasonably good. This may be explained by the presence of growth factors available in the medium including LY. Also it is possible that traces of these factors could have persisted in the bodies of the cells. In the subsequent passages, growth rates declined markedly. This was undoubtedly due to the progressive reduction of the necessary growth factors to a level below the minimum requirement of the cells. After four passages, the numbers of cells were insufficient for further sub-cultures. This must be explained by the assumption that certain essential growth requirements are supplied by calf serum. The amino-acids and vitamins included in the medium are insufficient for continued growth of the cells in the absence of calf serum.

In Experiment 5.8, the reason for the faster and better growth in the plastic flasks compared with that in the glass medical flats, might be explained because the infected cells can adhere more easily to the surface of the former. The higher growth rate and faster decline of cells in plastic Leighton tubes might be due to the smaller area with the consequent increased contact between cells bringing about clumping.

In Experiment 5.9, higher growth of schizont-infected cells in stationary culture, compared with that in stirred culture, is probably due to the cells being allowed to settle down on the surface. The numbers of non-viable cells in stirred cultures were low compared with those in stationary cultures. This might be due to the more rapid disintegration of dead cells by the action of the stirrer as non-viable cells disintegrate more readily. The stirring action appears to interfere adversely with growth of the cells but prolongs the plateau of cell growth.

In Experiment 5.10, the effect of per cent viability (%V.) of the seed-cells on rate of growth was investigated. The culture which was used as the source of cells for this experiment produced the pattern of viable, non-viable and %V. of the cultivated schizont-infected cells within eight days, Fig. 5.10. The viable cells which were cultivated with $10.0 \times 10^4$ cells per ml of medium increased in a sigmoid form during the first four days. Non-viable cells increased four fold in the first day and then varied little for the following two days. After three days the numbers of non-viable cells increased and after four days the proportion of non-viable to viable cells increased noticeably. Per cent viability of these cells appeared to be at a peak of about 95% on the third day of cultivation. Thereafter, this declined slowly, to the end of the culture period. It seems that within the first day of culture, old cells in the original seed die off, causing a fall in %V. on the first day. More rapid increase of non-viable cells occurs when viable cells pass the growth peak. In trial (a) of the experiment, the high growth of schizont-infected cells can be assumed to be caused by high %V. of the implanted cells since in all the cultures started with cells of medium %V. and low %V., conditions were the same. It was concluded from this experiment that when %V. of seed-cells was higher than 60, the cells grew satisfactorily and when the %V. was less than 50, the suspension culture developed more slowly. Therefore, it seems $5.0 \times 10^4$ and $10.0 \times 10^4$ would be sufficient numbers of seed-cells for the initiation of S.15 and S.3 cultures, respectively, provided the %V. is no less than 60. The passage intervals would then be four to five days. However, a higher %V. is obtainable with shorter interval passage.

In Experiment 5.11, higher growth in the cell cultures incubated at 37°C indicates that this temperature is most suitable, compared with the other temperatures included in the test.

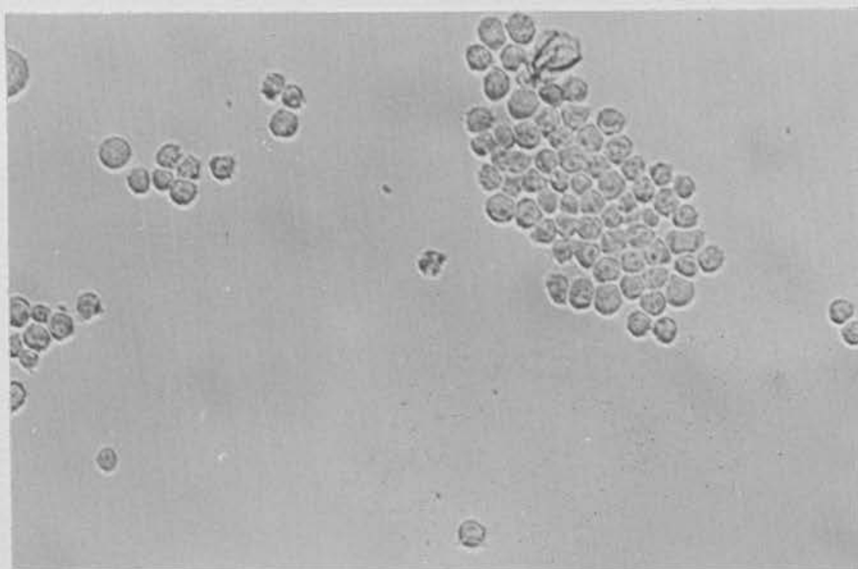
In Experiment 5.12, it was concluded that a feeder layer such as BHK cells could play an important role in the establishment of schizont-infected cells. This was shown by Hulliger (1965). This effect is most apparent in the early stages of isolation and adaptation to in vitro culture. Subsequently, a feeder layer is not necessary for growth. Figs. 5.15, 5.16 and 5.17 illustrate cultures with high %V., medium %V. and very low %V.

Fig. 5.13

Schizont-infected cells (S.15) in culture showing greater adherence to the surface of the vessel rather than clumping.

Fig. 5.14

Schizont-infected cells (S.3) in culture showing clump formation.



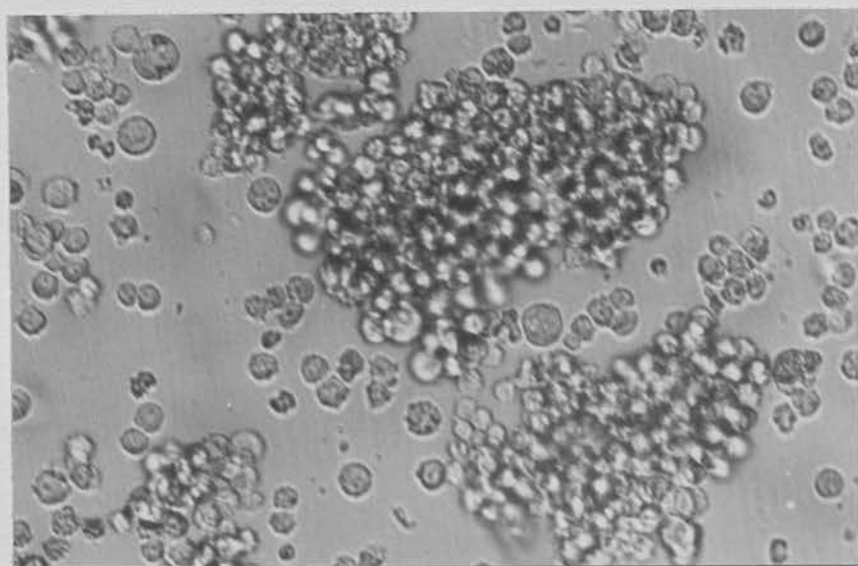


Fig. 5.15

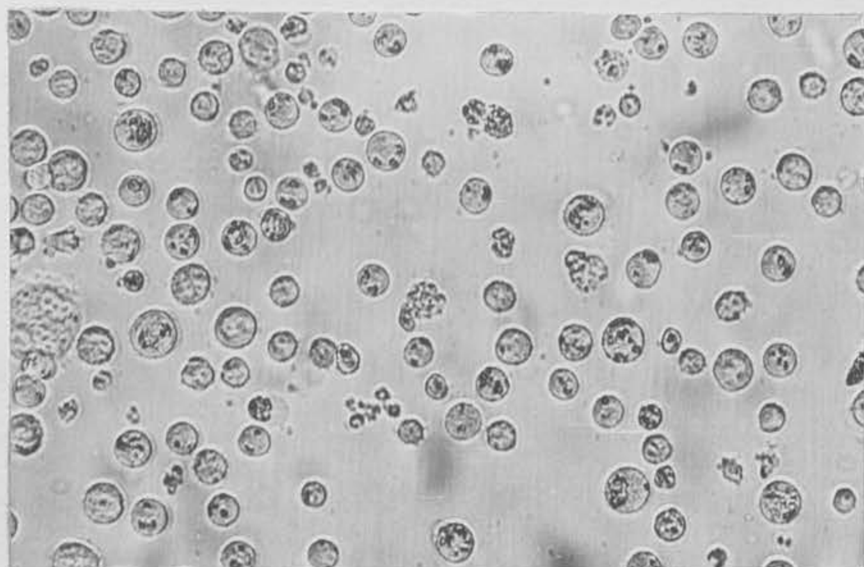
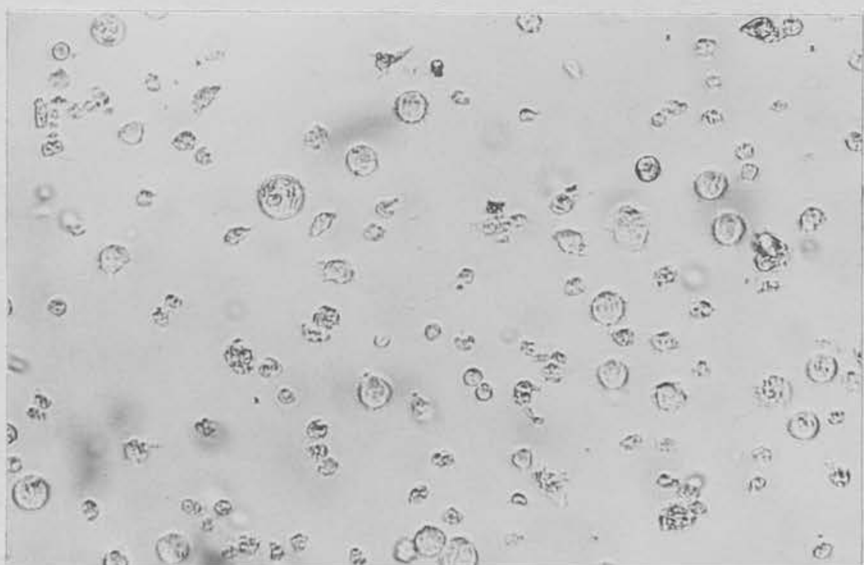
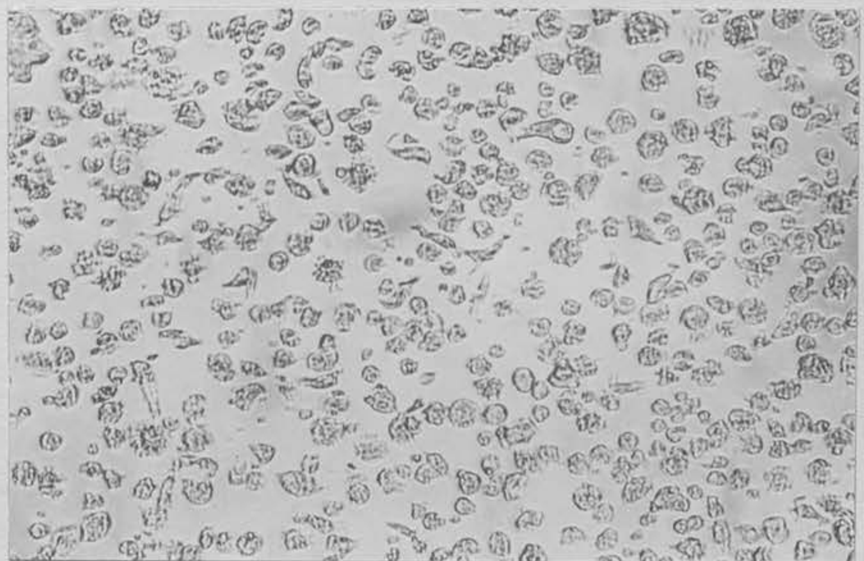
Schizont-infected cells in culture with high %V.

Fig. 5.16

Schizont-infected cells in culture with medium %V.

Fig. 5.17

Schizont-infected cells in culture with very low %V.

# CHAPTER 6

## MORPHOLOGICAL CHANGES IN THE DEVELOPMENT OF THE STAGES OF THEILERIA ANNULATA

In the review of literature it was explained that in established culture of Theileria annulata the parasite is in macroschizont form multiplying in the host cell and causing this to undergo mitosis. In the following experiments, morphology of the macroschizonts and some factors which cause their developmental changes are investigated.

A. Macroschizont-infected cells.

Experiment 6.1

Assessment of theilerial bodies in cultured infected lymphoid cells.

Theilerial bodies are referred to as a number of schizont particles in cytoplasm surrounded by a delicate membrane (Hulliger et al 1964). The numbers of these bodies are different in the host cells especially in T. annulata (S.15) in which boundaries of theilerial bodies are more distinguishable compared with those of T. annulata (S.3), (Figs. 6.1 and 6.2). To assess the percentage of Theileria-infected cells containing different numbers of these bodies, the following examination was carried out:- Schizont-infected lymphoid cells of T. annulata (S.15) were cultured in suspension. The medium used was Eagle's MEM with Hank's salts and ten per cent calf serum (CS) and supplemented with lactalbumin hydrolysate and yeast extract (LY) as the control medium. The pH of this medium was adjusted to 7.0 at

37°C using sodium bicarbonate and Hepes buffer (see Chapter 3). The medium was seeded with $5.0 \times 10^4$ and $10.0 \times 10^4$ cells per ml. The cultures were incubated at 37°C and sampled 24 hourly for staining preparations for six days. The stained linear smears (see Chapter 3) were scanned under oil immersion with a x50 objective and x15 ocular of a Wild microscope. The cells were chosen at random i.e., one out of every five cells and theilerial bodies were counted and recorded. In most of the stained slides the boundaries of theilerial bodies in the cell cytoplasm were not discernible thus, it was not possible to count these bodies in all preparations. Nevertheless, in each of ten smears, 100 of those cells containing theilerial bodies with a sharp and discernible membrane were counted and the percentages were calculated.

RESULTS

Ninety-five per cent of the host cells showed one theilerial body per cell and the remaining five per cent possessed from two to five as summarised in Table 6.1

Table 6.1

Per cent of *T. annulata*-infected cells with different numbers of theilerial bodies

| Nos. of theilerial bodies per cell | % of *Theileria*-infected cells |
|---|---|
| 1 | 95.0 |
| 2 | 3.2 |
| 3 | 1.1 |
| 4 | 0.5 |
| 5 | 0.2 |
| 6 | 0.0 |

Means of ten counts, each of 100 cells

Experiment 6.2

Assessment of schizont particles in cultured infected lymphoid cells.

Schizont-infected lymphoid cells (S.15) were cultured in the same way as previously described. Stained smears were prepared daily for six days. The cells which apparently contained one schizont particle only per cell were ignored, only those with more than one particle being considered for counts. A total of 500 cells which were chosen at random was scanned from daily preparations and the schizont particles in them were counted and recorded.

RESULTS

It was observed that the numbers of schizont particles per cell were variable for each day of culture. The minimum and maximum particles counted per cell varied from two to 114. The majority of the cells contained approximately five to 20 particles. A small number of the cells showed more than 50 to 80 particles, yet on rare occasions particles of less than five and more than 80 were observed. Minimum and maximum mean numbers of schizont particles per cell were 9.72 on the fourth day of cultivation and 16.16 on the sixth day and the overall mean number was 13.01. The data are given in Table 6.2.

In Experiments 6.1 and 6.2, division of theilerial bodies synchronous with cell mitosis was observed (Figs. 6.3 to 6.9). This corresponds with the observations of Wilde (1967) for *T. parva*.

Table 6.2

Assessment of schizont particles in cultured infected lymphoid cells

| Days of culture | Total Nos. of schizont particles in 500 cells per day | Average Nos. of schizont particles per cell |
|---|---|---|
| 1 | 6935 | 13.87 |
| 2 | 6150 | 12.30 |
| 3 | 5315 | 10.63 |
| 4 | 4860 | 9.72 |
| 5 | 7700 | 15.40 |
| 6 | 8080 | 16.16 |
| Overall mean No. of particles per cell | | 13.01 |

B. Attempts to produce further stages from the macroschizont-infected cells.

In this part two strains of *T. annulata*, S.15 and S.3 were used. The main purpose was to examine whether or not the macroschizonts undergo any further stages under various conditions *in vitro*, and if any further developmental changes occur, to what extent.

Before the experiments it seemed necessary to adopt a terminology by which to explain better the different stages of the parasite in culture.

TERMINOLOGY

The terminology adopted for this study is as described by Danskin and Wilde (1976b) with slight modifications to accord with

my observations on T. annulata as follows:-

Macroschizonts

These are groups of schizont particles, usually ten to 17, located in faint blue cytoplasm surrounded with a fine and delicate membrane. These chromatin particles stain reddish or purple and are irregular in shape, triangular, spurlike and round. The macroschizonts are similar in morphology in both the intra-cellular and the extra-cellular forms after rupture of the host cells. It was often observed that nuclear particles of macroschizonts of T. annulata (S.15) are large, triangular, tetrangular or irregular in shape and fewer in number compared with those of T. annulata (S.3) which are smaller and round in shape and more numerous (Figs. 6.1 and 6.2).

Transitional schizonts

These comprise numerous schizont particles, usually more than 25, in a bluish cytoplasm and membrane. The chromatin particles stain reddish and in some individuals dark reddish. Numbers of these chromatin particles seem to be in process of change into smaller round but more numerous bodies compared with macroschizont particles. The transitional schizonts can be found either in large blastoid cells or liberated from disrupted cells (Figs. 6.10 to 6.14).

Microschizonts

These appear as small,round schizont particles which stain densely and in stained smears most commonly are found disrupted from their host cells (Figs. 6.15 and 6.16).

Micromerozoites

These are single chromatin particles each enveloped in a small amount of blue cytoplasm. These originate from the dispersal of microschizonts as individual bodies when their precursors break away from ruptured host cells.

Piroplasms

These are the intra-erythrocytic forms of the micromerozoites which appear in different shapes as comma, rod, anaplasmoid and round and oval rings.

The following experiments were carried out to produce further stages from macroschizont-infected cells in culture.

Experiment 6.3

The initiation of new cultures using very high seed levels and the maintenance of old cultures.

(a) Control cultures. Two series of active cultures were set up, one from S.15 seeded with $5.0 \times 10^4$ cells per ml and the other from S.3 seeded with $10.0 \times 10^4$ cells per ml, both in duplicate. These cultures were initiated from active, three day-old cultures both with over 80 per cent viable cells. They were cultivated in 25 $cm^2$ plastic T-flasks incubated at 37°C and subcultured every three days regularly. Suspension cells were prepared from them on the first, second and third days of cultivation for three passages. Stained smears from these cultures which were made up of young and active cells were prepared and examined as controls.

(b) Overpopulated cultures. Other cultures were prepared as

previously described but seeded with four times as many cells per ml, as used in above cultures (a). The suspension cells were sampled from them one, two and three days after cultivation. The stained smears, considered as overpopulated, were prepared and examined.

(c) Old cultures. Two groups of cultures were set up as described in (a) above, one with subculturing into new culture vessels and the other in the initial culture vessels by reducing and adjusting the numbers of the seed cells in each passage to approximately the same level as in the originals. Both groups of cultures, however, were passaged every six days. The cultures were sampled at the fourth, fifth and sixth day of cultivation and stained smears were prepared from these samples. These were regarded as old cultures.

RESULTS

(a) In the stained slides from control cultures only macroschizonts were seen. Although in some cases cells were observed with more than 25 schizont particles, they were large in size and could not be regarded as transitional schizonts.

(b) Besides the macroschizonts, in rare cases the transitional schizonts were seen.

(c) The same as (b), rare transitional schizonts in cell cultures from new culture vessels and in the cultures from re-used vessels were seen. No microschizonts were observed in any of these trials, as summarised in Table 6.3.

Keys for the following tables are:

M.sch. = macroschizonts Tr.sch. = transitional schizonts

m.sch. = microschizonts m.m. = micromerozoites

p. = piroplasms

- = absent ? = doubtful + = rare

++ = frequent +++ = very frequent

Table 6.3

Further stages in overpopulated and old cultures.

| Cultures of schizont-infected cells | Sampling days | M. sch. | Tr. sch. | m. sch. |
|---|---|---|---|---|
| (a) Young, uncrowded control | 1 - 3 | +++ | - | - |
| (b) Overpopulated | 1 - 3 | +++ | + | - |
| (c) Old | 4 - 6 | +++ | + | - |
| (c) Old & from re-used flasks | 4 - 6 | +++ | + | - |

Experiment 6.4

The use of deep cultures.

Schizont-infected lymphoid cells, S.15 and S.3 were cultivated both in plastic Leighton tubes and in T-flasks. The tubes were set up at approximately 30$^{\circ}$ with the flat side upward. The flasks were sloped laterally at the same angle as above to let the cells sediment in round and curved corners of the culture vessels. Control cultures were incubated with the experimental cultures at horizontal position. Stained smears were prepared from these cultures once a day for four days.

RESULTS

In both cultures of S.15 and S.3 clumps of cells and fairly high numbers of dead cells were seen. The cultures of S.15 showed mostly macroschizonts and rare transitional forms without any microschizonts. The cultures of S.3 contained mostly macroschizonts, frequent transitional forms and rare microschizonts. These latter were seen on three occasions in twelve slides. In one slide prepared from the plastic Leighton tube after 48 hours one liberated schizont was observed in which there was a single nuclear particle (merozoite). For this observation, several more trials were repeated in deep culture using both strains but neither microschizonts nor micromerozoites were seen at all. The control cell cultures showed only macroschizonts (Table 6.4).

Table 6.4

Further stages in deep cultures.

| Strain | Clumps | M. sch. | Tr. sch. | m. sch. | m.m. |
|---|---|---|---|---|---|
| S.15 in Leighton tubes | + | +++ | + | - | - |
| S.15 in T-flasks | + | +++ | + | - | - |
| S.3 in Leighton tubes | ++ | +++ | ++ | + | ? |
| S.3 in T-flasks | ++ | +++ | ++ | - | - |
| Repeated both | +(S.15)<br>++(S.3) | +++ | +(S.15)<br>++(S.3) | - | - |
| Control both | - | +++ | - | - | - |

Experiment 6.5

The use of stirred cultures.

The infected cells were cultured in the three following ways

at $37^{o}C$:-

(a) Five ml aliquots of the cell culture were placed in 50 ml medical flats containing a two cm long magnet bar in each. These were placed on a magnetic stirrer[1] which rotated the magnet bar at 250 rpm.

(b) The same amounts of the cell culture were dispensed in 25 $cm^2$ T-flasks and these were fixed on the plate of a horizontal rotating shaker[2]. The speed was fixed in one trial at 50 rpm and in the other at 100 rpm.

(c) Two ml aliquots of the cell culture were distributed in 15 x 1.2 cm glass test tubes. These were inserted in the drum of a Roller Tube Tissue Culture Apparatus Model RT/1[3]. The rolling speed was approximately one revolution per eight minutes.

The cell suspensions were sampled at first after one, three and six hours and then 24 hourly up to four days. These samples and the controls were stained and scanned under the microscope.

RESULTS

(a) The cell cultures treated by the stirrer showed disrupted cells, frequent liberated macroschizonts and rare transitional schizonts but no microschizonts.

(b) The cells of shaken cultures were similar to those in the previous trial with rare transitional schizonts and with vacuoles in some of the host cells and in theilerial bodies.

(c) The cells from rolling tubes showed macroschizonts, clumps

- - - - - - - - - - - - - - - -

(1) Townson and Mercer Ltd., Croydon, England.

(2) Rotates R/200/2, Luckham Ltd., Burgess Hill, Sussex.

(3) Matburn Ltd., London.

of cells especially with S.3, no transitional schizonts nor microschizonts. The control cultures contained only macroschizonts (Table 6.5).

Table 6.5

Further stages in the stirred cultures.

| Cultures from : | Clumps | M.sch. | Tr.sch. | m.sch. |
|---|---|---|---|---|
| (a) Stirred medical flats | - | +++ | +(S.15) ++(S.3) | - |
| (b) Shaken flasks | - | +++ | +(S.15) ++(S.3) | - |
| (c) Rolling tubes | + | +++ | - | - |
| Control | - | +++ | - | - |

Experiment 6.6

The use of growth media with partial deficiency of nutrient constituents.

In this experiment the schizont-infected lymphoid cells were cultured in several growth media, with some partial nutritive deficiencies as follows:-

(a) Eagle's MEM with Hank's supplemented with CS and LY as the control growth medium (See Chapter 3). The process of cultivation was as described in Experiment 6.1.

(b) The medium as (a) but without the supplement LY.

(c) The medium as (a) but without calf serum.

(d) The medium as (a) but without glutamine.

(e) A modified Eagle's medium without glucose. In this medium the combined Hank's balanced salt solutions (A and B) also CS which

contain glucose were substituted with PBS and 0.5g per cent lactalbumin hydrolysate and 0.1g per cent yeast extract which do not contain glucose (see Chapter 8).

(f) A medium made up with 0.5g per cent lactalbumin hydrolysate and 0.1g per cent yeast extract (LY) and ten per cent CS in Hank's BSS but without additional amino-acids and vitamins.

(g) Same medium as (f) above with Earle's BSS in place of Hank's. The cells cultured in the above media were sampled daily for four days and stained smears were examined.

RESULTS

(a) The cells in the control medium showed good growth resulting in typical macroschizonts and no transitional schizonts.

(b) The medium without LY showed cells with macroschizonts but no further stages.

(c) The cells in the medium without CS showed poor growth with macroschizonts and rare transitional schizonts.

(d) Macroschizonts and rare transitional schizonts were observed in this trial.

(e) The cells did not grow well in this medium and the samples prepared from the cultures on the first three days showed only macroschizonts in the cells.

(f) and (g) The viable cells declined in numbers and only macroschizonts were seen in the cells. A few cells showed 20 schizont particles which were large and could not be referred to as transitional schizonts. No microschizonts were confirmed in any of these media, as shown in Table 6.6.

Table 6.6

The use of growth media with partial nutritive deficiencies.

| Trials | Media | M. sch. | Tr. sch. | m. sch. |
|---|---|---|---|---|
| a | Eagle's MEM + CS + LY (Control) | +++ | - | - |
| b | Control + CS - LY | +++ | - | - |
| c | Control - CS + LY | +++ | + | - |
| d | Control - glutamine + CS + LY | +++ | + | - |
| e | PBS - glucose & some salts - CS + LY | +++ | - | - |
| f | Hank's salts + CS + LY | +++ | - | - |
| g | Earle's salts + CS + LY | +++ | - | - |

Experiment 6.7

Cultivation in increased $CO_2$ tension.

This experiment was conducted with the idea that gradually increased pressure of $CO_2$ and decreased oxygen gases produced in the culture might stimulate the macroschizonts to change into microschizonts. Duplicate cultures of the two strains, S.15 and S.3 were each dispensed in the following containers along with control cultures (e) and incubated:- (a) five Bijou bottles, (b) five 3.0 ml round bottom plastic tubes, (c) five 2.5 ml ampoules and (d) five 1 x 10 mm capillary tubes. The Bijou and plastic tubes were filled to the top with suspension culture and were screwed tightly. The ampoules and the capillaries were almost filled and sealed over a gas flame. All were set in the upright position except the capillaries which were kept horizontal. Duplicates of the cultures were used daily for staining and examination for five days.

## RESULTS

(a) The cells showed macroschizonts, rare transitional schizonts and no microschizonts.

(b) The cells showed macroschizonts, frequent transitional schizonts and doubtful microschizonts in S.3 cultures only on days four and five.

(c) The cells showed macroschizonts, rare transitional schizonts but no microschizonts.

(d) The cells from the capillaries demonstrated macroschizonts, frequent transitional schizonts but no typical microschizonts. In all cultures clump formation was seen particularly in the cultures of S.3, also many dead cells especially in the ampoules and capillaries were observed.

(e) The control culture was as in previous experiment. Trial (b) was repeated several times with calf washed rbc to indicate the presence of any further stages, but no micromerozoites or piroplasms were found, (Table 6.7).

Table 6.7

Cultivation in increased $CO_2$ tension.

| Trials | Culture container | Clumps | M. sch. | Tr. sch. | m. sch. | m.m. | p. |
|---|---|---|---|---|---|---|---|
| a | Bijou | + | +++ | + | - | | |
| b | Plastic tube | + | +++ | ++ | ? | - | |
| b | Plastic tube + Bov. rbc | + | +++ | ++ | + | - | - |
| c | Ampoule | + | +++ | + | - | - | |
| d | Capillary | + | +++ | ++ | - | - | |
| e | Control, T-flask | - | +++ | - | - | - | |

Experiment 6.8

Cultivation in media with various pH values.

Three batches of Eagle's MEM plus CS and LY (Chapter 3), were prepared and were adjusted to pH (a) 8.0, (b) 6.0 and (c) 7.0, using Hepes buffer and sodium bicarbonate. Schizont-infected cells of S.15 and S.3 were seeded at rates of $5.0 \times 10^4$ and $10.0 \times 10^4$ cells per ml of medium respectively in these three different pH media. These were incubated at 37°C and cell suspensions were prepared from them after six hours and then 24 hourly for five days and examined.

RESULTS

(a) The cells in the alkaline culture, pH 8.0 diminished gradually in four days so that on the fifth day most of the cells were non-viable. The slides prepared in the first four days from these cells showed only macroschizonts.

(b) The cells cultured in the medium with pH 6.0 showed fast growth, macroschizonts and rare and frequent transitional forms as well as doubtful microschizonts with S.3. This trial with pH 6.0 was repeated several times, also with the addition of freshly prepared bovine washed rbc to the cultures. As a result, frequent transitional forms and rare microschizonts were observed after six hours, overnight and 48 hours, all with S.3.

(c) The cells from the control showed only macroschizonts (Table 6.8).

Experiment 6.9

Cultivation at various temperatures.

Several trials were carried out in this experiment.

(a) Schizont-infected cell cultures, S.15 and S.3, were kept in

Table 6.8

Cultivation at various pH values.

| Trials | pH | M.sch. | Tr.sch. | m.sch. | m.m. | p. |
|---|---|---|---|---|---|---|
| a | 8.0 | +++ | - | - | - | |
| b | 6.0 | +++ | +(S.15)<br>++ (S.3) | -(S.15)<br>? (S.3) | - | |
| b | 6.0+<br>rbc | +++ | +(S.15)<br>++(S.3) | ?(S.15)<br>+ (S.3) | - | - |
| c | 7.0 | +++ | - | - | - | |

the refrigerator at approximately 4°C and stained smears were prepared after three, six and 18 hours.

(b) and (c) The cell cultures were kept at 18°C and 25°C respectively for three days and stained smears were prepared daily.

(d) The cultures of schizont-infected cells were incubated at 38, 39 and 40°C respectively for four days. Stained smears were prepared from them 24 hourly. Similar cultures were set up in a shaker water bath with the temperature adjusted to 38, 39 and 40°C. Stained smears were prepared from each for four days.

(e) Cell cultures were incubated at 41°C and 42°C for four days. These were sampled for staining and examination after three and six hours and then 24 hourly until the fourth day when all the cells died off.

(f) Control cultures were set up at 37°C.

RESULTS

(a) The infected cells kept in the refrigerator at three hours and six hours showed only macroschizonts, while after 24 hours

approximately 50 per cent of the initial cells died and the remaining viable cells contained macroschizonts only, with no further stages.

(b) and (c) The cells incubated at 18°C and 25°C showed a rapid decline and macroschizonts only.

(d) The cells incubated at 38°C and 39°C either stationary or agitated showed macroschizonts and transitional schizonts with S.3. No microschizonts were seen. In the cells cultured at 40°C, in both stationary and shaken cultures, macroschizonts, transitional schizonts, microschizonts and no micromerozoites were seen. The cultures shaken in the water bath showed also many ruptured cells and free macroschizonts as well as vacuoles in intact cells.

(e) The cells incubated at 41°C and 42°C after three and six hours contained rounded-up macroschizont particles. After 24 hours, frequent transitional schizonts appeared along with a decreased number of intact viable cells. On the first day and during the following days typical microschizonts were observed, while the transitional schizonts were increasing and macroschizonts were decreasing in number. The microschizonts appeared almost always along with ruptured cells. No micromerozoites were seen.

(f) The control culture cells showed only macroschizonts.

Trials (d) at the teperature 40°C and (e) at the temperatures 41 and 42°C were repeated with the addition of bovine washed rbc but no further stages, either micromerozoite or piroplasm, were seen (see Table 6.9).

Experiment 6.10

Cultivation with the addition of foreign cells.

In this experiment schizont-infected cells were cultured in

Table 6.9

Cultivation at various temperatures.

| Trials | Temperature (°C) | M.sch. | Tr.sch. | m.sch. | m.m. | p. |
|---|---|---|---|---|---|---|
| a | 4 | +++ | - | - | - | |
| b | 18 | +++ | - | - | - | |
| c | 25 | +++ | - | - | - | |
| d | 38 | +++ | -(S.15) +(S.3) | - | - | |
| d | 39 | +++ | -(S.15) +(S.3) | - | - | |
| d | 40 | +++ | + | -(S.15) +(S.3) | - | |
| d | 40 + Bov. rbc | +++ | ++ | + | - | |
| e | 41 | +++ | ++ | ?(S.15) +(S.3) | - | |
| e | 41 + Bov. rbc | +++ | ++ | + | - | - |
| e | 42 | +++ | + | + | - | |
| e | 42 + Bov. rbc | +++ | ++ | + | - | - |
| f | Control 37 | +++ | - | - | - | |

association with the following foreign cells:-

(a) BHK cells were versenated and cultured at the rate of $1.0 \times 10^5$ cells per ml of medium with the same number of schizont-infected cells.

(b) The same number as before of green African monkey, (Vero) cells were mixed with schizont-infected cultures.

(c) Horse white blood cells and (d) rabbit WBC were mixed in the cultures at the rate of $5.0 \times 10^5$ cells per ml of medium.

(e) Sheep WBC and sheep washed rbc were applied to the cultures, the former up to $5.0 \times 10^5$ cells per ml and the latter up to approximately $3.0 \times 10^6$ cells per ml of medium.

These mixed cultures along with the control cultures were incubated at 37°C for five days and stained smears were prepared from them and examined daily.

RESULTS

(a) Association of BHK cells was found favourable to the growth of schizont-infected cells. These however, showed only macroschizonts.

(b) The infected cells cultured with Vero cells showed the same picture as in trial (a) above. No transitional schizonts nor microschizonts were seen in this trial.

(c) and (d) The macroschizonts did not undergo any further development when cultured with horse and rabbit WBC. Only doubtful and rare transitional forms were seen with S.15 and S.3 respectively.

(e) Association of sheep WBC with the cultures resulted in the appearance of rare transitional forms. In the cultures to which sheep washed rbc had been added, macroschizonts, rare transitional schizonts and disrupted cells were seen. The sheep rbc, in a few cases, were found surrounding schizont-infected cells and some appeared to be entering these cells. However, no microschizonts were found.

(f) The control cells did not produce any further stages from the macroschizonts (see Table 6.10).

Table 6.10

Cultivation with the addition of foreign cells.

| Trials | Culture (Schizont-infected cells = control) | M. sch. | Tr.sch. | m. sch. | m.m. |
|---|---|---|---|---|---|
| f | Control | +++ | - | - | - |
| a | " + BHK cells | +++ | - | - | - |
| b | " + Vero cells | +++ | - | - | - |
| c | " + horse WBC | +++ | ?(S.15) +(S.3) | - | - |
| d | " + rabbit WBC | +++ | ?(S.15) +(S.3) | - | - |
| e | " + sheep WBC | +++ | + | - | - |
| e | " + sheep rbc | +++ | + | - | - |

Experiment 6.11

Cultivation with the addition of normal bovine WBC, lymph node cells and rbc.

(a) Normal bovine lymphocytes, leucocytes and lymph node cells were prepared as described in Chapter 3. In many trials $1.0 \times 10^6$ per ml of these cells were mixed and cultured with optimal numbers of schizont-infected cells of S.15 and S.3 as in the above experiment.

(b) Corresponding cultures of the schizont-infected cells were mixed and cultured with normal bovine washed rbc at the rate of $3.0 \times 10^6$ cells per ml of medium. The cultures were set up in both flat and round bottom culture vessels and incubated at 37°C for five days. These suspension cultures were sampled daily along with a control culture and stained smears were examined daily. Trial (a) was repeated with the addition of washed bovine rbc.

RESULTS

The results are tabulated in Table 6.11.

(a) Stained smears showed macroschizonts, rare transitional forms as well as doubtful microschizonts with S.3 only. In repetition of Trial (a) with the addition of bovine rbc, transitional forms and microschizonts increased further in numbers. No further stages were seen.

(b) Besides the macroschizonts, rare transitional forms as well as microschizonts were observed. These two forms were found more in the round bottom culture vessels. No micromerozoites nor piroplasms, however, were seen.

Table 6.11

Cultivation with the addition of normal bovine WBC, lymph node cells and rbc.

| Trial | Culture (Schizont-infected cells = control) | M. sch. | Tr. Sch. | m.sch. | m.m. | p. |
|---|---|---|---|---|---|---|
| | Control | +++ | - | - | - | |
| | Control + bov. lymphocytes | +++ | + | - | - | |
| | " + bov. lymphocytes + bov. rbc | +++ | ++ | ?(S.15) +(S.3) | - | - |
| a | " + bov. leucocytes | +++ | + | -(S.15) ?(S.3) | - | |
| | " + bov. leucocytes + bov. rbc | +++ | ++ | ?(S.15) +(S.3) | - | - |
| | " + bov. lymph node cells | +++ | + | -(S.15) ?(S.3) | - | |
| | " + bov. lymph node cells + bov. rbc | +++ | ++ | ?(S.15) +(S.3) | - | - |
| b | " + bov. rbc | +++ | + | ?(S.15) +(S.3) | - | - |

Experiment 6.12

Cultivation with the addition of normal lymph node extract (LNE).

Extract of normal bovine lymph node (LNE) was prepared as described in Chapter 3. Final concentrations of one, five, ten and 20 per cent v/v of LNE were applied and the ten per cent was chosen and used. The cell cultures were set up in both shallow and deep positions, as described in Experiment 6.4. The cultures were sampled 24 hourly for five days and stained smears were prepared and examined. Control cultures were included.

RESULTS

Macroschizonts, transitional forms and microschizonts but no micromerozoites were observed. It appeared that high concentrations of LNE were toxic to the cells so that the higher the concentration used the more infected cells died. The transitional schizonts were found more frequently than in Experiment 6.11. Microschizonts appeared earlier in the deep culture than in the shallow one. They were seen more frequently in the cultures of S.3 than in the cultures of S.15. The control cell cultures were as before (see Table 6.12).

Experiment 6.13

Cultivation with the addition of lymph node extract (LNE) and rbc of normal cattle.

This experiment was a repetition of the previous experiment to examine if the microschizonts produced in culture containing LNE undergo further stages of development and eventually infect the rbc. The LNE was added to the cultures of schizont-infected

Table 6.12

Cultivation with the addition of normal bovine lymph node extract (LNE)

| Culture | M. sch. | Tr. sch. | m. sch. | m.m. |
|---|---|---|---|---|
| Schizont-infected cells (control) | +++ | - | - | - |
| Cells of S.15 + LNE in shallow culture | +++ | + | ? | - |
| Cells of S.15 + LNE in deep culture | +++ | ++ | + | - |
| Cells of S.3 + LNE in shallow culture | +++ | ++ | + | - |
| Cells of S.3 + LNE in deep culture | +++ | ++ | ++ | - |

cells at the rate of ten per cent v/v. Freshly prepared washed rbc at the rate of $3.0 \times 10^6$ per ml were added and the cultures were set up in deep positions. This experiment was carried out repeatedly using S.15 and S.3 of T. annulata as well as T. parva for comparison as some similarity between this and S.3 was seen. Stained smears were prepared daily for five days in each trial and were examined.

RESULTS

Stained smears displayed macroschizonts, disrupted host cells, transitional forms and microschizonts. In the preparations from T. annulata-infected cultures, rare cases of rbc with small bodies were seen but, they were not confirmed to be piroplasms. No micromerozoites were seen. In the stained smears prepared from T. parva-infected cultures besides the microschizonts rare micromerozoites and doubtful piroplasms were found as summarised in Table 6.13.

Table 6.13

Cultivation with the addition of lymph node extract and rbc of normal cattle.

| Culture | M. sch. | Tr. sch. | m. sch. | m.m. | p. |
|---|---|---|---|---|---|
| T. annulata-infected cells (control) | +++ | - | - | - | |
| T. annulata-infected cells + LNE + rbc | +++ | ++ | ++ | - | - |
| T. parva-infected cells + LNE + rbc | +++ | ++ | ++ | + | ? |

Experiment 6.14

Cultivation with the addition of calf lymph and rbc of normal cattle.

In this experiment, to the cultures of schizont-infected cells, ten per cent calf lymph[1] and approximately $3.0 \times 10^6$ per ml bovine washed rbc were added. This experiment was carried out to compare S.3 of T. annulata-infected cells with T. parva-infected cells. The cultures were sampled daily for five days for examination.

RESULTS

In stained smears of T. annulata-infected cells, macroschizonts, disrupted cells, transitional forms and microschizonts were observed but no micromerozoites. The stained slides prepared from T. parva-infected cells, in addition to the above stages, showed rare micromerozoites in a single cytoplasmic envelope (Table 6.14).

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(1) Calf lymph was obtained from Danskin and Wilde (1976).

Table 6.14

Cultivation with the addition of calf lymph and rbc of normal cattle.

| Culture | M. sch. | Tr. sch. | m. sch. | m.m. | p. |
|---|---|---|---|---|---|
| T. annulata-infected cells (control) | +++ | - | - | - | |
| T. annulata-infected cells + calf lymph + rbc | +++ | ++ | ++ | - | - |
| T. parva-infected cells + calf lymph + rbc | +++ | ++ | ++ | + | ? |

DISCUSSION

In Experiment 6.1, the percentage counts showed that a majority of 95 per cent of the host cells contained one theilerial body. This indicates that a single body per cell is the usual form in regularly growing cells. The small minority of the cells with more than one theilerial body per cell might be assumed to be due to factors such as traumas, rapid osmotic exchange and phagocytic function affecting the theilerial body, in the cell cytoplasm.

In Experiment 6.2, more than 3,000 schizont-infected cells were scanned at various intervals of one to six days incubation. The average number of schizont particles per cell was 13.01, the majority lying between five and 20. In some cells, the numbers of particles were high, frequently exceeding 50 per cell. This, which was usually observed on the fifth and sixth days of incubation, was possibly due to the slowing down of the mitosis of the host cells while the schizont particles continued multiplication at the same rate. Hulliger *et al* (1964) demonstrated

the variation of schizont particles per cell as between 14 and 17, which is similar to the above ranges. Hulliger (1965) re-examined the number of schizont particles in T. annulata-infected cell culture and calculated that the average number of these particles per cell was 13. Although the cultures by the previous authors were associated with BHK cells, their results are confirmed by the present investigation, carried out with pure suspension cultures.

In attempts to produce further stages from the macroschizonts, throughout twelve experiments including many trials, no further stages appeared beyond the macroschizont in routine conditions. The appearance of transitional forms in over-populated and old cultures (Experiment 6.3) and in deep culture (Experiment 6.4) might have been due to the fact that the numbers of cells per ml of medium were too high thus causing conditions unfavourable for cell growth.

The production of rare and frequent transitional schizonts (in S.15 and S.3 respectively) in stirred cultures, could have been due to retardation of growth of the host cells due to agitation. Frequent liberated theilerial bodies observed in the stained preparations probably resulted from stirring and shaking which disrupted the large and mature cells. Agitation could be the reason for the appearance of some vacuoles in both cells and theilerial bodies. In the control cultures this did not happen (Experiment 6.5).

Various nutritive deficiencies were compared in Experiment 6.6 and none of them produced any microschizonts or further stages. The rare transitional forms which occurred in some cases might have been due to the difference in multiplication rate of

host cells and schizont particles. Hulliger *et al* (1966) also stated that in a partially deficient medium they failed to produce microschizonts in *T. parva* culture.

In Experiment 6.7, cultures in sealed and tightly closed vessels, in some cases, produced transitional forms varying from rare to frequent. This might have been the result of combined factors of $CO_2$ pressure, oxygen decrease, deep culture and over-population which were exerted by this method. When this trial was repeated with the addition of normal calf washed rbc, doubtful and rare microschizonts appeared in cultures of S.15 and S.3 respectively but no micromerozoite nor piroplasm was found. Hulliger *et al* (1966) reported that by varying oxygen tension they failed to produce microschizonts in *T. parva* culture.

The medium of pH 6.0 in Experiment 6.8 proved to be a factor in accelerating the growth rate resulting in microschizonts in disrupted cells. This developmental change occurred quickly, as within six hours transitional forms and microschizonts were observed.

In Experiment 6.9, the effect of various temperatures on the macroschizonts were investigated. The low range of temperatures was not associated with any development of the schizonts and this is as might be expected since at the temperatures used, *viz.* 4°C, 18°C and 25°C, there is no multiplication of host cells. At the higher range of temperatures, 40°C, 41°C and 42°C, rapid multiplication occurred with the appearance of transitional forms and microschizonts. The latter, in both strains, were rare only. However, when washed bovine rbc were added to these cultures at the higher temperatures the proportion of transitional forms and microschizonts was increased but, still, no micromerozoites nor

piroplasms were seen.

Experiment 6.10, as can be seen in Table 6.10, showed that the addition of foreign cells to cultures which were otherwise maintained routinely, made no significant difference to the development of forms beyond the macroschizont except that, with the addition of horse and rabbit white blood cells, and of sheep WBC and rbc some transitional forms appeared.

In Experiment 6.11, it appeared that bovine WBC and lymph node cells as well as rbc were associated with the appearance of transitional forms and microschizonts of the parasite. When bovine rbc were added to cultures in which this effect was noticed, the effect appeared to be more marked but in none of the cultures were micromerozoites or piroplasms seen. The part played by bovine rbc became clear when these alone were applied in Trial (b) and resulted in the production of rare transitional forms and microschizonts.

A similar development of transitional forms and a small number of microschizonts were demonstrated in Experiments 6.12 and 6.13 in which lymph node extract (LNE) was added to the culture medium. There was an indication that with S.3 the deep culture of macroschizonts in the presence of LNE showed a greater tendency to produce microschizonts than in the other cultures. The addition of bovine rbc to deep cultures with LNE had little noticeable effect on T. annulata schizonts but with T. parva a few micromerozoites and possible piroplasms were seen. The toxic effect of LNE was demonstrated in Experiments 6.12 and 6.13 when disrupted cells were often observed. This effect probably caused the macroschizonts to change into microschizonts.

In Experiment 6.14 in which calf lymph was used, the results

were the same as in Experiment 6.13. The production of microschizonts along with disruption of the cells could be explained as due to the toxic effect of the calf lymph. Danskin and Wilde (1976a and 1976b) demonstrated the toxicity of the calf lymph on *T. parva*-infected cells. These authors by using the calf lymph in the culture of *T. parva*-infected lymphoid cells and then with the addition of normal bovine washed rbc to the culture succeeded to produce microschizonts, micromerozoites and eventually piroplasms in the rbc after the latter was added to the culture.

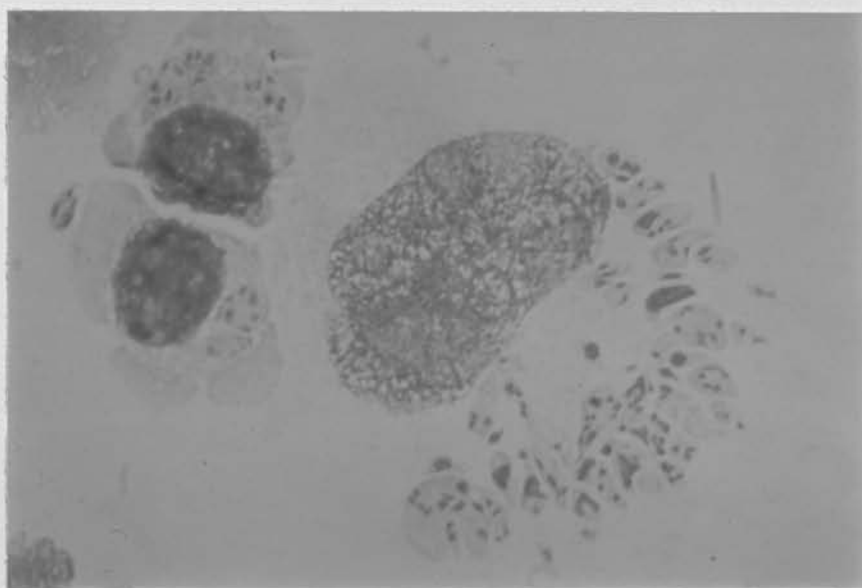
Throughout previous experiments, in no cases did macromerozoites or micromerozoites break off *T. annulata* macroschizonts, whether these were cultured in enriched media or under various unfavourable conditions. This observation agrees with the statement "one simply does not see the macroschizonts breaking up into uninucleate macromerozoites" (Brocklesby 1970). In this series of experiments in which cultivation was carried out in various conditions described with the object of exerting pressures on the developing schizont to the extent of causing it to produce the definitive piroplasmic stage, no predictable method of bringing this about was found.

Fig. 6.1

Schizont-infected cells showing theilerial bodies in the cells and liberated schizonts.

Fig. 6.2

A schizont-infected cell containing numerous schizont particles before completion of division.



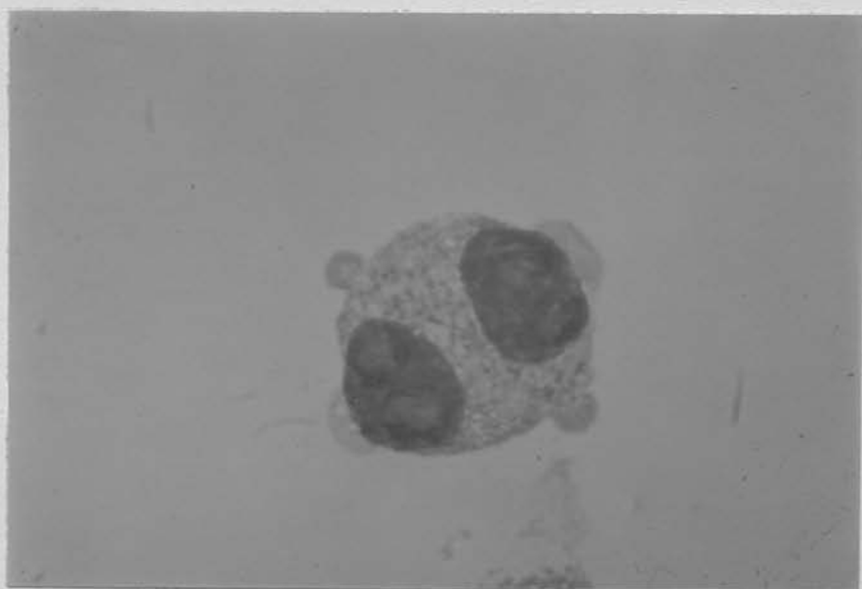
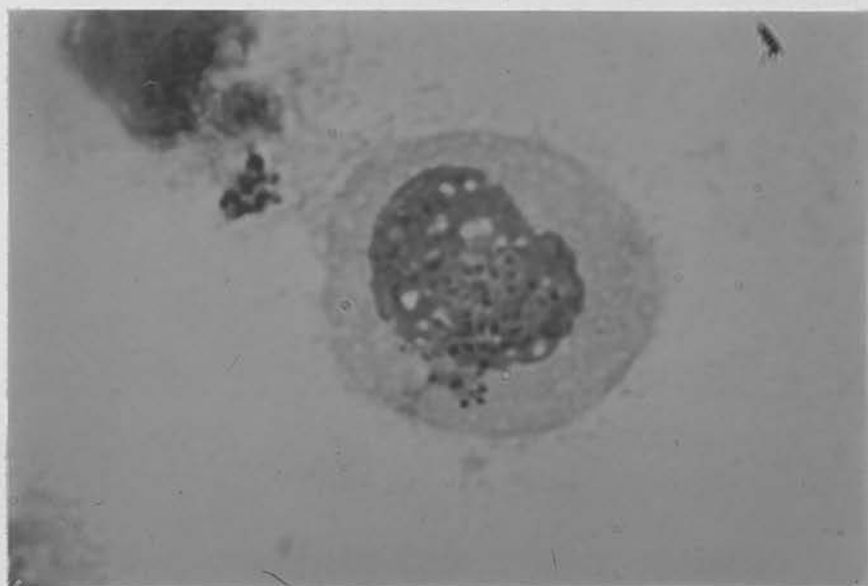


Fig. 6.3

A schizont-infected cell. Schizonts are seen in association with formation of chromosomes (interphase/prophase).

Fig. 6.4

Schizont-infected cells. Schizonts associated with chromosomes (early metaphase).



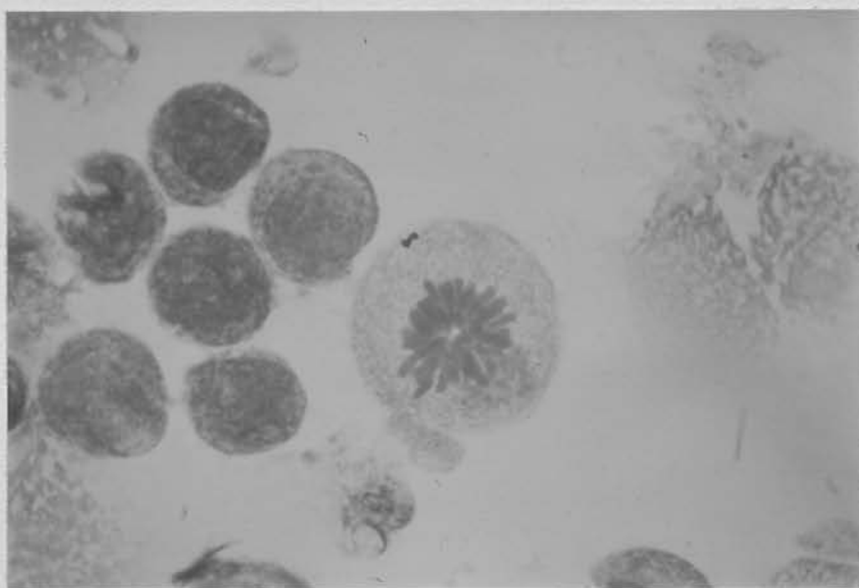
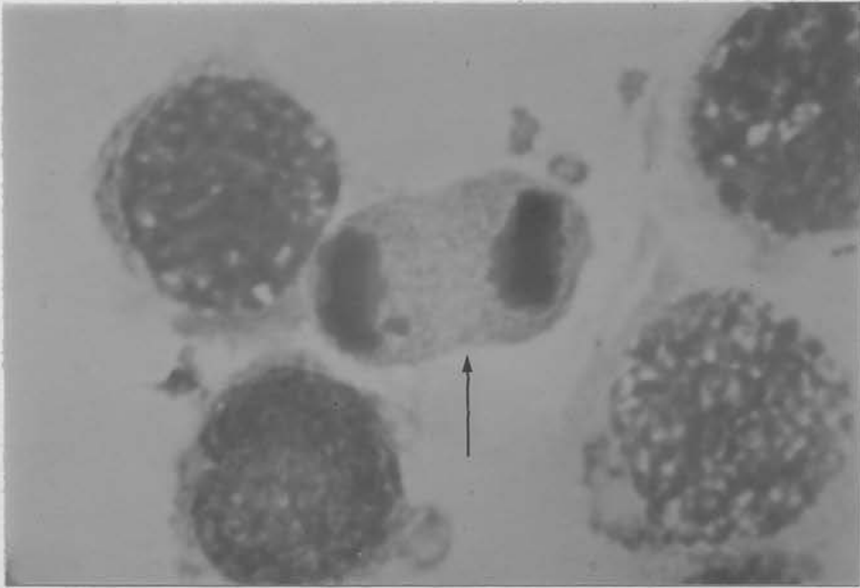


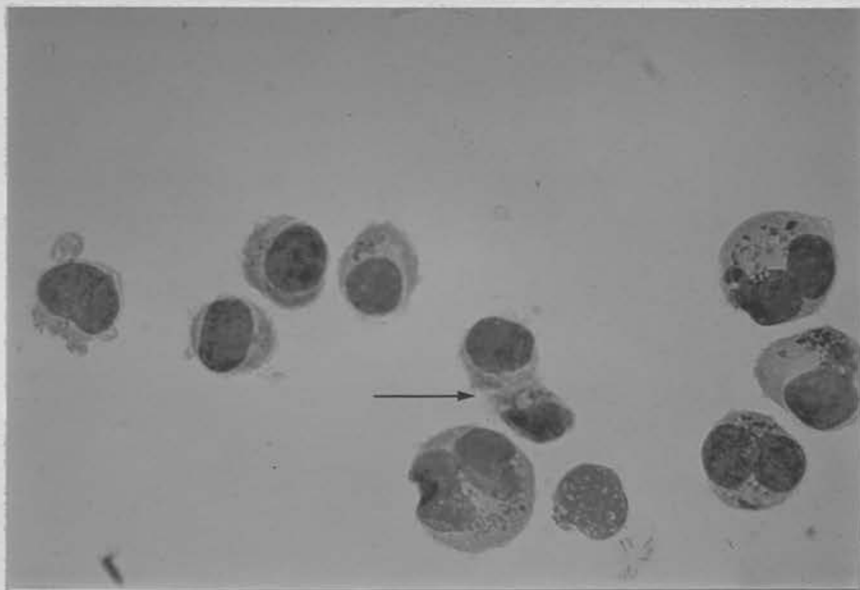
Fig. 6.5

A schizont-infected cell (anaphase/telophase).

Fig. 6.6

Schizont-infected cells. Telophase is shown arrowed in one of the cells.



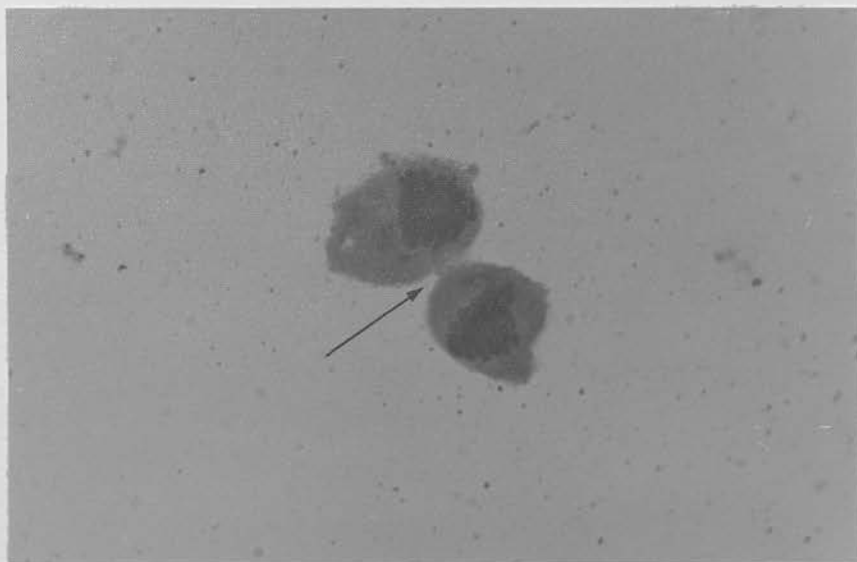


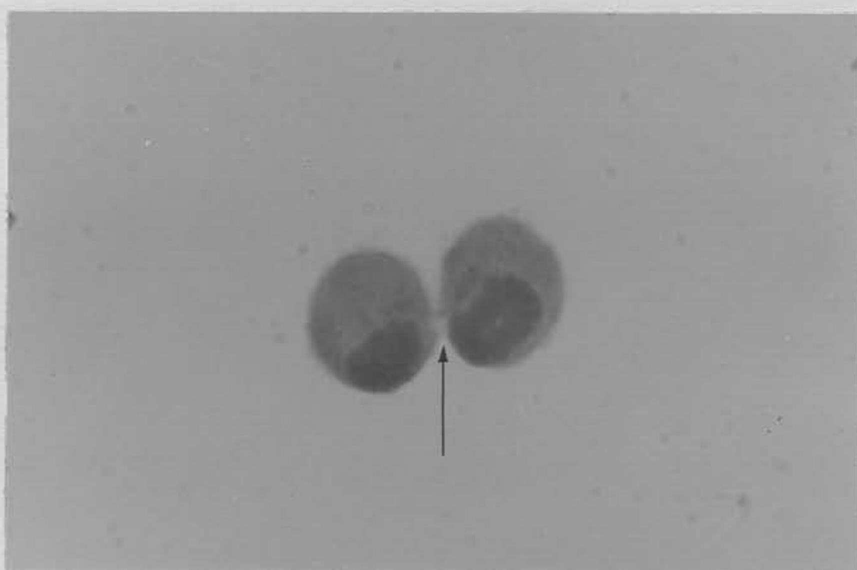
Figs. 6.7 and 6.8

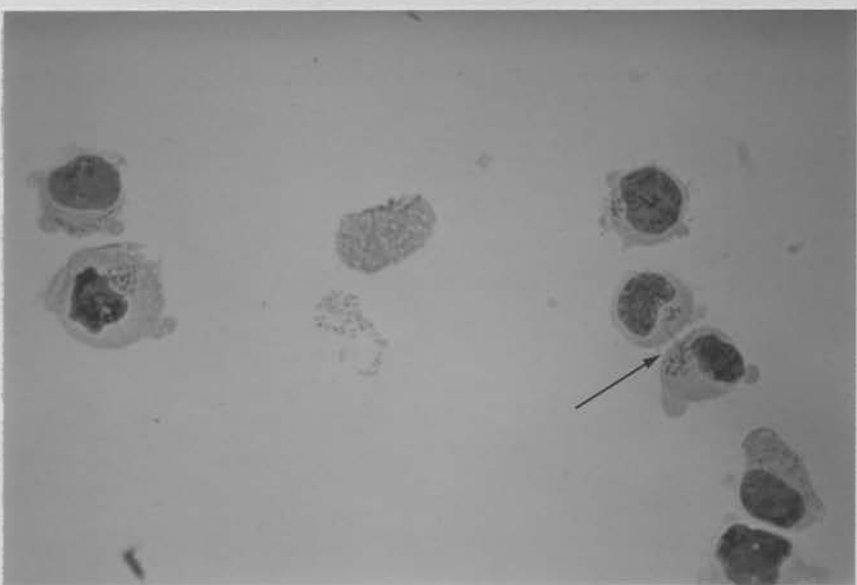
Schizont-infected cells at telophase. The schizont strands are indicated by arrows.

Fig. 6.9

Schizont-infected cells showing telophase or reconstitution. The schizonts which are distributed to two daughter cells are shown.

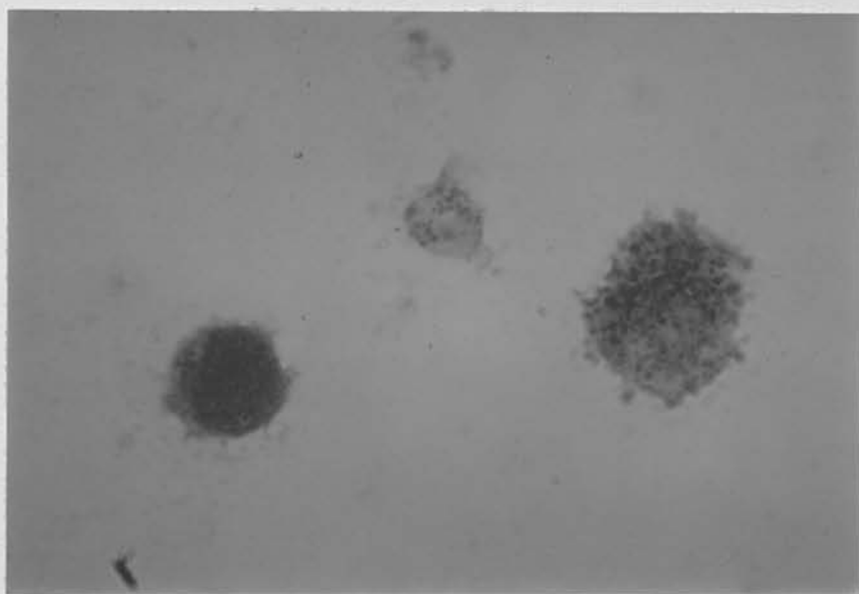


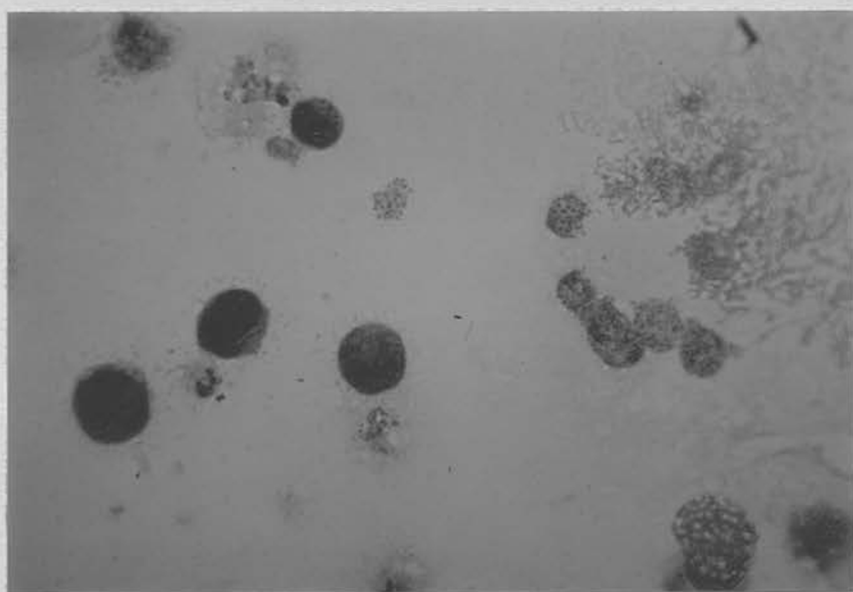




Figs. 6.10 and 6.11

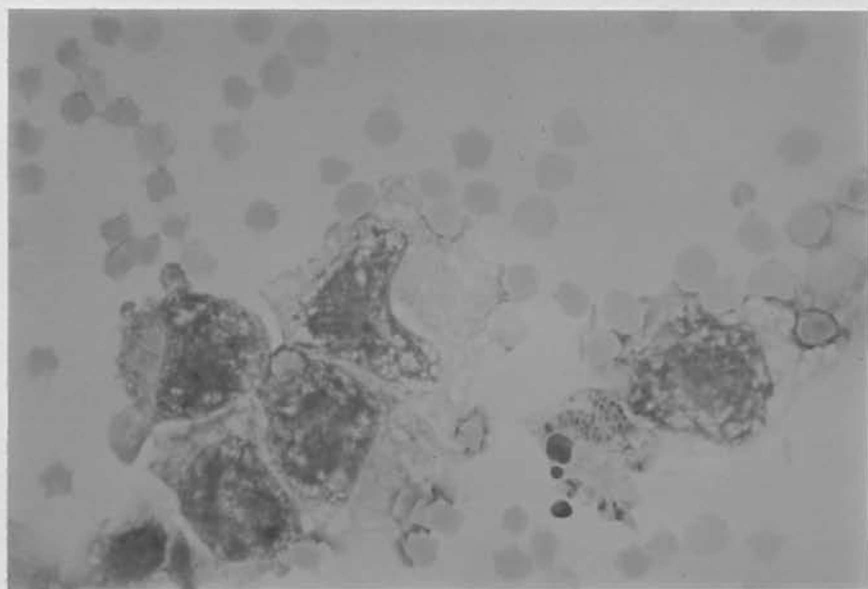
Schizont-infected cells showing transitional forms and microschizonts.

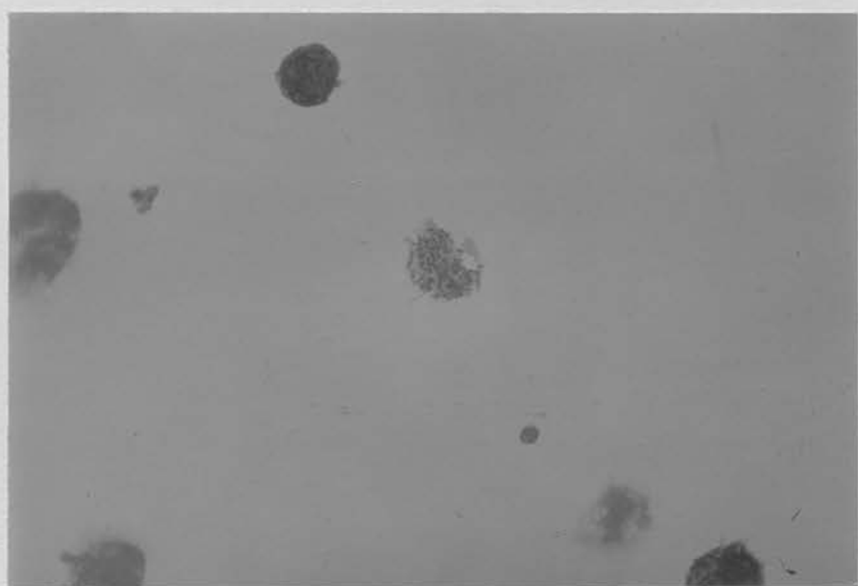


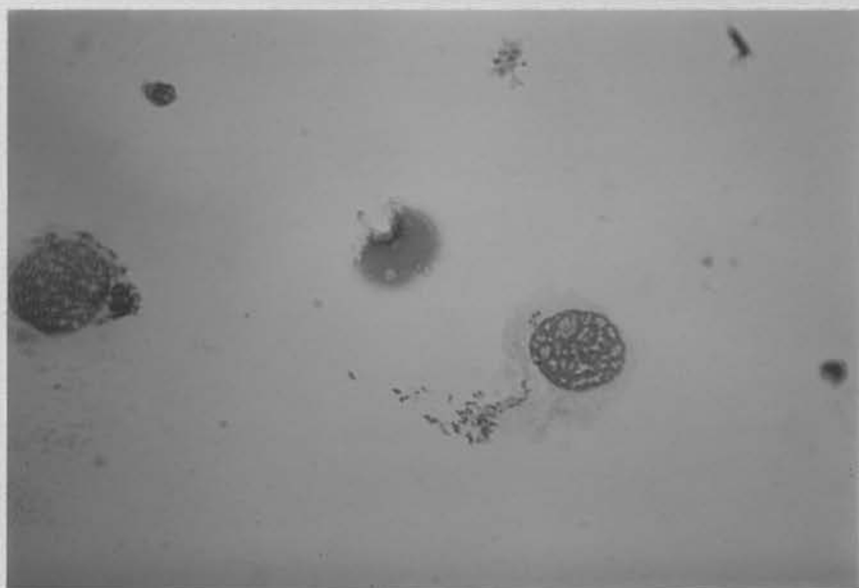


Figs. 6.12, 6.13 and 6.14

Schizont-infected cells showing the macroschizont, transitional forms and microschizont.

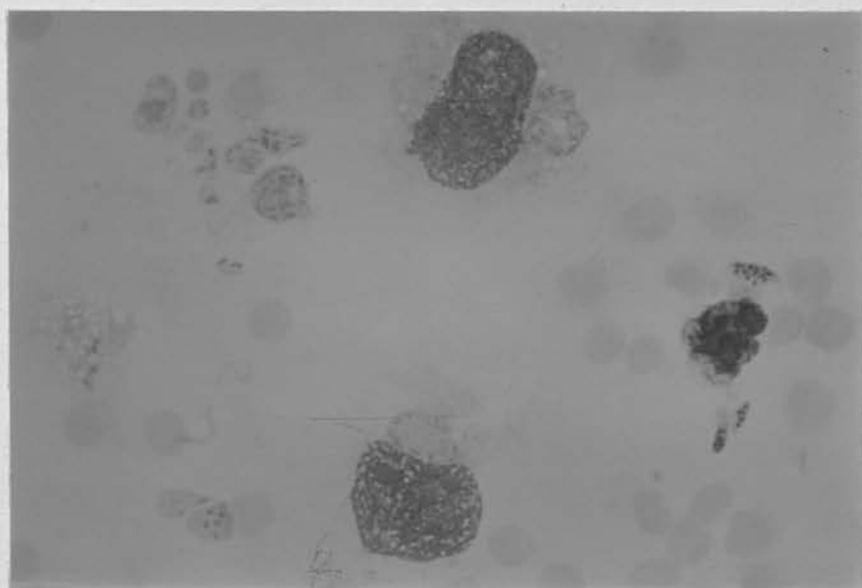


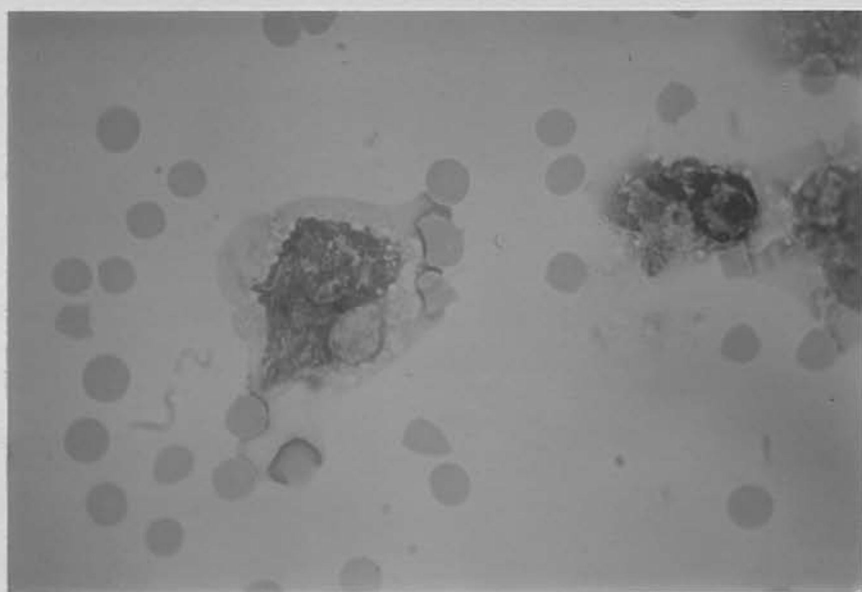




Figs. 6.15 and 6.16

Schizont-infected cells showing the macroschizonts (theilerial bodies) inside the host cells and liberated, and typical microschizonts.





# CHAPTER 7

## ATTEMPTS TO TRANSFER *THEILERIA ANNULATA* SCHIZONTS TO NORMAL UNINFECTED CELLS

Early workers on East Coast fever assumed that in the propagation of *T. parva* the macroschizont, at a certain stage in its cycle, left the host cell and broke up into merozoites which were then capable of infecting other host cells. This assumption was generally accepted although Reichenow (1940) suggested that the propagation might be by binary fission of the host cell and simultaneous division of the parasite. Hulliger *et al* (1964), using a tissue culture system, showed that a form of propagation of *Theileria* species takes place in which the macroschizont enters into the process of mitosis of the host cell so that one part of the schizont body is transferred to one daughter cell and the other to the other daughter cell. These authors stated that this process also takes place *in vivo*. Since Hulliger *et al* (1964) published this work, it has been generally accepted that this process is probably the main method of propagation of the macroschizont. It has, however, been acknowledged that this process might not be exclusive but that possibly, in addition, schizonts freed from the host cell might invade other lymphocytes of the host animal and so continue growth and multiplication. Wilde *et al* (1966) considered that such schizonts (Koch's bodies) freed from the host cells, played no further part in the life cycle.

A conference considering the immunology of *T. parva*, held in Nairobi in December 1974, suggested that the breakout of macroschizonts and invasion of other cells might well take place and

contribute to the process of propagation. It was suggested by this conference that further work should be carried out on this aspect of the theilerial life cycle (Wilde 1975).

The work described in this chapter was, therefore, designed to explore, in vitro, the validity or otherwise of this hypothesis.

The methods adopted were based on the principle of bringing together, in vitro, lymphocytes transformed by the presence of macroschizonts and fresh lymphocytes from healthy uninfected cattle. In view of the difficulty of establishing cultures of uninfected lymphocytes (see Chapter 8), it was decided that it would be necessary to add fresh lymphocytes to established cultures of macroschizont-infected cells. In order to differentiate between the culture cells and the added cells, a form of labelling would be necessary. As the established cultures were in male cells, it was decided that labelling could be dependent on karyotyping and so all fresh lymphocytes were obtained from female animals.

The normal uninfected cells used in the following experiments were: (a) pure lymphocytes separated from blood, (b) blood leucocytes, (c) lymph node cells and (d) spleen cells; all from healthy female cattle. It was thought that any possible in vitro transmission of macroschizonts to uninfected cells could occur only in two ways. First, by the invasion of fresh lymphocytes by free schizonts, in the manner originally believed to occur in vivo (Brocklesby 1970). Second, by the phagocytosis by fresh healthy lymphocytes of schizonts either within their host cells or free (Wilde et al 1966). If the latter occurred, it was necessary to demonstrate that the normal cells used were capable of phagocytosis. Experiment 7.1 was designed to demonstrate this. Prior to every experiment a viability test was carried out and it was shown that

the normal cells used were 74.0 to 98.0 per cent viable.

Experiment 7.1

Phagocytic control test using normal cattle cells against opsonised sheep red blood cells.

There is general agreement that normal cells of blood, lymph node and spleen can phagocytose inert particles and foreign cells, (Odajima and Sonoda 1971; Begemann and Rastetter 1972; Weir 1974; Schalm, Jain and Carroll 1975 and Ling and Kay 1975). Nevertheless, a control test was performed in order to show that the normal cells used were phagocytically active. The normal cells were incubated in suspension and in shallow cultures in which a certain proportion of cells attached to the floor of the vessels. A one per cent suspension of washed sheep red blood cells (SRBC) was prepared in PBS. Five ml of this were mixed with an equal volume of a 1:1200 dilution of rabbit anti-sheep erythrocyte antiserum (RASES)(1) and incubated for 30 minutes. The suspension was then centrifuged and washed with PBS three times and the final sedimented SRBC were resuspended in five ml of PBS. The antibody coated SRBC, so produced, were added to the normal cells in the ratio of one to five v/v. These were incubated at 37°C and samples were made into smears for microscopic examination at one and two hours, respectively. By these means the phagocytosis of all four types of cells, viz. blood lymphocytes/leucocytes, lymph node cells and spleen cells was examined.

RESULTS

The opsonised SRBC were seen to be phagocytosed by the

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(1) Burroughs Wellcome Co. Ltd., London.

uninfected cells in all cases, Figs. 7.1 and 7.2. As the phagocytic potential of these healthy cells had been demonstrated, it appeared possible that schizonts, either freed from their host cells or still within them, could be similarly phagocytosed and the schizonts then might become established in new host cells.

A series of experiments was, therefore, planned in order to expose schizonts either free or in their host cells to suspensions of living healthy uninfected bovine cells from the blood, lymph nodes and spleen.

In the first two experiments, 7.2 and 7.3, the schizonts were brought into contact with the fresh cells without any attempt being made to release them from their host cells. In normally active cultures of schizont-infected lymphoblasts a proportion of the schizonts is found freed in the medium so that in experiments where such cultures are brought into contact with suspended normal cells, the opportunity exists for phagocytosis by these cells of free schizonts and schizont-infected lymphoblasts.

Experiment 7.2

Exposure of schizont-infected lymphoblasts to normal cells over a long period by the repeated addition of fresh uninfected cells.

The normal cells were suspended at a rate of $1.5 \times 10^6$ cells per ml in Eagle's MEM, with Hank's salts and supplemented with CS and LY, (see Chapter 3). Schizont-infected cells were added to these cultures at levels not exceeding $5.0 \times 10^4$ cells per ml of medium, and the mixtures were incubated at $37^oC$. Every three or four days cell counts were made and stained smears were prepared. At the same time the cultures were adjusted to contain $5.0 \times 10^4$

schizont-infected cells per ml by the substitution of a proportion of the cultures with fresh medium. Also, every seven days freshly prepared normal cells were added to the mixed cultures to bring the non-infected viable cells up to a level of $1.5 \times 10^6$ per ml of the suspension. Chromosome preparations were carried out from these cultures every seven days, and examined. These continuous mixed cultures were maintained for eight weeks. Over this period several times duplicate cultures in which cell monolayers had formed, were versenated. Stained smears were prepared from them and also chromosome preparations were examined.

RESULTS

Stained smears showed many cells in which schizonts were not distinguishable. These were presumed to be the normal cells. All karyotype analyses indicated mitosis of male cells only i.e., the original schizont-infected cells.

Experiment 7.3

Exposure to normal cells of schizont-infected cells using old cultures containing many cells and a high proportion of free schizonts.

Six day old cultures which had been initiated with suspensions containing $10.0 \times 10^4$ and $20.0 \times 10^4$ schizont-infected cells respectively, were pooled for examination and found to have a cell viability of 56 per cent and a large number of free schizonts, (Fig. 7.3). Several mixed suspensions were then set up for incubation containing $1.5 \times 10^6$ normal uninfected cells and $5.0 \times 10^4$ schizont-infected cells with the corresponding free schizonts. These were maintained for three days after which fresh medium was

substituted for a proportion of the suspensions to bring the schizont-infected cells to the original level of $5.0 \times 10^4$ per ml and the cultures were maintained for a further three days. Throughout this period, at intervals of 24 hours, samples were taken from the cultures and subjected to viability counts, examination of stained smears and chromosome analyses. Some cells at the end of the six days were attached to the vessels; these were removed by versenation and examined in the same way.

RESULTS

Stained smears demonstrated a proportion of cells without schizonts, presumably the normal cells; in the remainder, schizonts were distinguishable. Chromosome analyses showed mitosis only in infected male cells.

Experiment 7.4

Exposure to normal cells of schizont-infected cells ruptured by means of some hypotonic solutions.

In the two previous experiments in which self-freed schizonts from a long period of sub-culturing and from old cultures were exposed to the normal cells, no infection of new cells occurred. It was decided to increase the number of freed schizonts by rupturing infected cells. Thus an increased number of freed schizonts could be exposed to the normal cells. The following ten experiments were planned for this purpose, using various methods.

Hypotonic solutions of Hank's BSS, Earle's BSS, PBS, sodium chloride, potassium chloride and ammonium chloride were used in many preliminary trials and the first (Hank's BSS) was chosen as the most satisfactory. Schizont-infected cells were centrifuged

and sedimented cells were resuspended in three hypotonic strengths of Hank's BSS, x0.9, x0.8 and x0.6 being incubated for 60, 30 and five minutes, respectively. Afterwards, the isotonicities of the suspensions were restored immediately by the addition of required amounts of x10 Hank's BSS. Samples of these suspensions were used for viability tests and staining. As a result 59.17 per cent of the cells treated with the strength of x0.8 for 30 minutes were ruptured and the rest remained viable. These cells were exposed to the normal cells for four days at 37°C. The other combinations of the hypotonic solutions and appropriate times produced either too low (18.75 per cent) or too high (90.84 per cent) cell disruption respectively. These were not regarded as satisfactory.

RESULTS

Chromosome analysis showed mitoses only in infected male cells.

Experiment 7.5

Exposure to normal cells of schizont-infected cells ruptured by detergents.

Two detergents, sodium dodecyl sulphate[1] and Triton X-100 (octyl phenoxypolyethoxyethanol)[2] were used at the dilutions of 1:5000 and 1:500, respectively. The infected cells were mixed with these detergents for five minutes and were then washed and centrifuged three times in order to remove all traces of the detergents. The infected cells were disrupted to the extent of

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(1) BDH Chemicals Ltd., England.

(2) Sigma Chemical Co., England.

47.0 per cent and 32.0 per cent by these two treatments respectively. The treated cells were mixed with normal cells at the rates previously described. Incubation and examinations of stained smears and chromosome preparations were carried out.

RESULTS

Karyotype analysis showed mitoses only in infected male cells.

Experiment 7.6

Exposure to normal cells of schizont-infected cells disrupted by freezing/thawing method.

The infected cell suspension was frozen at -150°C for five minutes without any cryoprotectants in two ml aliquots and then it was thawed in a water bath at 38°C. The freezing/thawing was carried out once, twice and thrice and cell counts and stained smears showed that single freezing/thawing resulted in 88.5 per cent cell disruption. Single freezing/thawing was, therefore, used for the production of free schizonts which were exposed to normal cells as before.

RESULTS

Chromosome analysis showed mitoses in schizont-infected male cells only.

Experiment 7.7

Exposure to normal cells of schizont-infected cells disrupted with rabbit anti-bovine lymphocyte antiserum (RABLS).

Various concentrations of RABLS in schizont-infected cell

suspensions for different exposure times were tested and, as a result, the use of two per cent v/v RABLS in the cell suspensions for 30 minutes was chosen to rupture the cells. The treated cells were then centrifuged and washed with PBS three times. In this way 73.0 per cent of the cells were ruptured and the rest remained viable. The infected cells so disrupted were mixed with the suspension of normal cells as before.

RESULTS

Chromosome analysis showed mitoses only in infected male cells.

Experiment 7.8

Exposure to normal cells of schizont-infected cells ruptured by ultrasonic disintegration.

To rupture the infected cells by sonication, a 100 Watt ultrasonic disintegrator(1) was used. This was tuned for four to eight microns peak-to-peak on the amplitude meter for 30 to 60 seconds. Four and six micron amplitudes for 45 seconds and 30 seconds were chosen, respectively. By the use of these two treatments, 58.0 per cent and 62.0 per cent of the cells were ruptured respectively and the remaining cells were intact. These ruptured cells were exposed to the normal cells, and incubated as before.

RESULTS

Karyotype analysis showed mitoses in infected male cells only.

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(1) MSE Ltd., London.

Experiment 7.9

Exposure to normal cells of schizont-infected cells disrupted by mechanical means.

Tests were applied to culture suspensions to determine the most satisfactory method of disrupting the infected cells by mechanical means. These included (a) agitation using pipette and syringe, (b) grinding in a Tenbruk grinder both lightly and vigorously and (c) agitation with glass beads in a Universal bottle by means of a Whirlimixer.

As a result, the method chosen was the agitation with glass beads as in (c), for a period of one minute, which produced disruption to the extent of 47.0 to 52.0 per cent. The cells so disrupted were exposed to the normal cells as in the previous experiments.

RESULTS

Chromosome analysis showed mitoses only in infected male cells.

Experiment 7.10

Exposure of separated schizonts from disrupted infected cells, to normal cells.

An attempt was made to separate the freed schizonts produced as in Experiment 7.9 from the intact cells in the suspension. Preliminary tests were made using (a) filtration through layers of filter paper (Whatman No. 1)[1], (b) filtration through the millipore prefilter pad, (c) filtration through 3.0 µm and 5.0µm millipore membranes, (d) filtration through a sintered glass

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(1) Balston Ltd., England.

filter (Sinta Glass No. 3)(1) and (e) separation using Lymphoprep with centrifugation as described in Chapter 3.

Stained smears from all the filtrates and deposits produced by centrifugation of the filtrates were examined. None of these methods produced theilerial bodies completely separated from intact cells. In methods (a), (b), (c) and (d) only theilerial particles and debris were found. By method (e) theilerial bodies were produced in the filtrate, but these were mixed with numbers of intact infected cells.

Table 7.1 shows the result of preliminary tests using method (e).

Table 7.1

Separation of schizonts from disrupted infected cells, using gradient centrifugation.

| Time of centri-fugation (minutes) | No. of viable cells per ml, x10$^3$ | No. of non-viable cells per ml, x10$^3$ | Per cent viability of sedi-mented cells | Abundance of theilerial bodies per microscopic field |
|---|---|---|---|---|
| 3 | 15.0 | 580.0 | 2.52 | very frequent |
| 5 | 45.0 | 620.0 | 6.76 | very frequent |
| 10 | 225.0 | 685.0 | 24.72 | frequent |
| 15 | 360.0 | 740.0 | 32.72 | rare |

The tabulated values are duplicate counts of only sedimented cells.

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(1) Pyrex Ltd., England.

Cell counts of suspension culture before disruption were:

| Viable cells per ml, $x10^3$ | Non-viable cells per ml, $x10^3$ | Per cent viability |
|---|---|---|
| 1100.0 | 260.0 | 80.88 |

Cell counts of suspension culture after disruption were:

| Viable cells per ml, $x10^3$ | Non-viable cells per ml, $x10^3$ | Per cent viability |
|---|---|---|
| 480.0 | 735.0 | 39.5 |

From these,it was decided to use the suspension following three minutes of centrifugation because this gave the highest proportion of theilerial bodies to viable intact cells.

In addition, filtrates from 5.0µm millipore filtration were used as these appeared to contain schizont particles separated from theilerial bodies. The filtrates prepared in this experiment were exposed to the normal cells in the mixed suspension as before.

RESULTS

Chromosome analysis showed mitoses only in infected male cells.

Experiment 7.11

Exposure to normal cells of ruptured and opsonised infected cells.

The infected cells were ruptured to approximately 50 per cent as in (c), Experiment 7.9. Sedimented cells were suspended in PBS,

to which one per cent RABLS was added. The suspension was incubated at 37°C for 30 minutes. This was centrifuged and washed with PBS three times and finally mixed and exposed to the normal cells as before. Incubation and examinations were carried out in the usual way.

RESULTS

Karyotype analysis showed mitoses only in infected male cells.

Experiment 7.12

Exposure of ruptured schizont-infected cells to PHA-activated normal leucocytes.

Normal female leucocytes were prepared and suspended in growth medium at a rate of 1.5 x $10^6$ cells per ml of medium, to which 1.6 per cent v/v PHA was added. The cell suspension was incubated at 37°C for three days, by which time the cells were actively in mitosis and presumably phagocytic. To this suspension were added infected cell suspensions in which the cells had been disrupted by method (c) of Experiment 7.9. Incubation and examinations were carried out as before.

RESULTS

Karyotype analysis showed mitoses only in schizont-infected male cells.

Experiment 7.13

Exposure to PHA-activated leucocytes of schizont-infected cells disrupted and opsonised.

Normal female leucocytes were activated using PHA and

incubated as described in the previous experiment for three days. Schizont-infected cells were first disrupted as in the previous experiment and then opsonised using RABLS as before. Several aliquots of this suspension were added to culture vessels in which PHA-activated leucocytes had already been incubated. Incubation continued and chromosome preparations were made after two hours and then 24 hourly up to three days.

RESULTS

Karyotype analysis showed mitoses only in male cells, i.e. the original schizont-infected cells, the schizonts being associated with the chromosomes, Figs. 3.6 and 3.7. In two Experiments, 7.12 and 7.13, in which PHA-activated normal cells were used, female chromosomes were also observed but there were no schizonts associated with them, Fig. 3.9.

In Experiment 7.11, when opsonised schizont-infected cells were exposed to normal cells, stained smears from mixed and deep cultured cells showed one eosinophil containing a theilerial body with four schizont particles. This was seen in no other preparations nor in further observations.

Experiment 7.14

Phagocytic control test using schizont-infected cells against opsonised sheep red blood cells (SRBC).

Having done the previous experiments in which firstly the phagocytic function of the normal cells was proved (Experiment 7.1) and, secondly, no cases of infection of fresh cells was observed, (Experiments 7.2 to 7.13), it was decided to test the phagocytic function of the schizont-infected cells in order to compare it with

the phagocytic function of the normal cells. This was carried out in the following experiment.

Washed SRBC were prepared, coated with RASES and resuspended in PBS, as described in Experiment 7.1. This suspension was added to a schizont-infected cell suspension at the ratio of one to five and incubated for two hours. This mixed cell suspension was sampled and stained smears were prepared after one and two hours of incubation.

RESULTS

In Experiment 7.14, very marked phagocytosis was observed. Schizont-infected cells phagocytosed opsonised SRBC abundantly and in several cases up to four SRBC were seen in one infected cell, Figs. 7.4 to 7.7.

This phagocytosis was much more dramatic than that seen in Experiment 7.1.

Experiment 7.15

Attempts to infect healthy calves with separated schizont particles of three strains of T. annulata.

In order to examine whether or not the schizont particles separated from the culture were alive and infective to susceptible cattle this experiment was designed. Extra-cellular schizonts were often observed in stained smears prepared from schizont-infected cultures. To separate these free schizonts from the host cells several preliminary trials were carried out, by filtering the suspension culture through 3.0, 5.0 and 8.0μm millipore membranes. The filtrates were spun down and stained smears prepared from the deposits were examined. It was shown that the filtrates from 3.0

and 5.0μm membranes were cell-free and contained small bodies characterised as schizont particles. The filtrate obtained using 8.0μm membrane was not cell-free and contained some small intact cells in addition to the schizont particles; therefore this was not used in the experiment. The membrane of 5.0μm porosity was chosen as it produced cell-free schizont particles in larger numbers than those passing through the 3.0μm membrane. This experiment was carried out in three trials using three strains of T.annulata, S.15, S.3 and S.19.

(a) One hundred ml of a suspension of schizont-infected cells of S.15, containing $5.0 \times 10^5$ cells per ml, were stirred by means of a magnetic stirrer[1] at 250 rpm for ten minutes. This was performed to obtain more extra-cellular schizonts from mature and old infected cells. The suspension was then filtered through a 5.0μm millipore filter and the filtrate was spun down at 1000 G for 15 minutes. Stained smears from deposited material showed many schizont-particles. Neither intact cells nor theilerial bodies were observed. The deposited material was resuspended in five ml growth medium and then injected subcutaneously into a normal Holstein calf aged eight months. The calf was kept under control for four weeks. The body temperature was measured twice daily and stained smears from peripheral blood were examined daily. Stained smears were prepared from prescapular lymph node and examined from the 14th day of inoculation, daily or as appropriate.

(b) One hundred ml of schizont-infected cell culture, S.3 were treated as in (a) above. The final deposited material was resuspended in five ml growth medium and inoculated subcutaneously

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(1) Gallencamp Co. Ltd., England.

into a healthy Holstein calf aged seven months. The same examinations were carried out with this calf as in (a) above.

(c) One hundred ml suspension culture of schizont-infected cells, S.19, were used as in the previous trials above. Sedimented material obtained from filtrate of the suspension culture was resuspended in five ml growth medium. This was inoculated subcutaneously into a normal Holstein calf, aged seven months. Observations and examinations were carried out with this animal as in (a) and (b) above.

RESULTS

None of the three calves showed any thermal reaction, nor were parasites seen in blood or lymph node smears.

The calves, when inoculated with the homologous strains of T. annulata, using two ml of culture ($4.0 \times 10^6$ cells), all showed typical theileriosis infections.

DISCUSSION

The normal cells used throughout the previous experiments were tested and found to be between 74.0 and 98.0 per cent viable and to be phagocytically active. Thus the methods for separating the cells had not affected their viability and phagocytic potential (Odajima and Sonoda 1971).

Phagocytosis of freed schizonts by normal cells could not be demonstrated because absolute separation of freed schizonts from remaining intact cells was not possible. This is confirmed by a similar study performed by Malmquist et al (1970), who failed to separate the extra-cellular form of T. parva from infected cultures using centrifugation and filtration.

Therefore, it was decided to utilise phagocytosis by normal cells on the assumption that, if free schizonts were phagocytosed and established themselves in these cells, they might undergo mitosis and produce continuous culture which could be proved karyotypically.

The schizonts were, therefore, exposed to the normal cells, either in low numbers as self-freed or in high numbers by disruption of the infected cells. The question was whether or not the free schizonts were alive and infective to fresh lymphocytes. Various methods, i.e. deep cultures, shallow cultures with normal cells attached to floors of the vessels, long period subculturing, short period exposure with opsonisation of the infected cells and activation of the normal cells, were tried. Karyotype analyses made in every experiment showed mitosis in male cells only except that in two experiments in which the normal cells were activated by PHA, female chromosomes were seen but these were not associated with the schizonts and in subsequent subcultures when PHA was no longer used female cells were not seen in mitosis.

Therefore, it can be assumed that the schizonts, in vitro, are so closely cell-associated that either immediately after separation from their host cells or at most before they can be phagocytosed, they die off (Wilde et al 1966).

The phagocytic control test in Experiment 7.14 indicated that schizont-infected cells exhibited a much stronger phagocytic potential against SRBC than did the normal cells used in Experiment 7.1. This could indicate that when the two types of cells are mixed, they could be in phagocytic competition in which the schizont-infected cells would be more likely to phagocytose than to be phagocytosed in vitro. In some instances, normal

lymphocytes were actually engulfed by schizont-infected lymphocytes (Fig. 7.8). This provides strongly presumptive evidence that there is no transfer of schizonts or schizont-infected lymphocytes in the viable potentially propagating state, at least, in vitro.

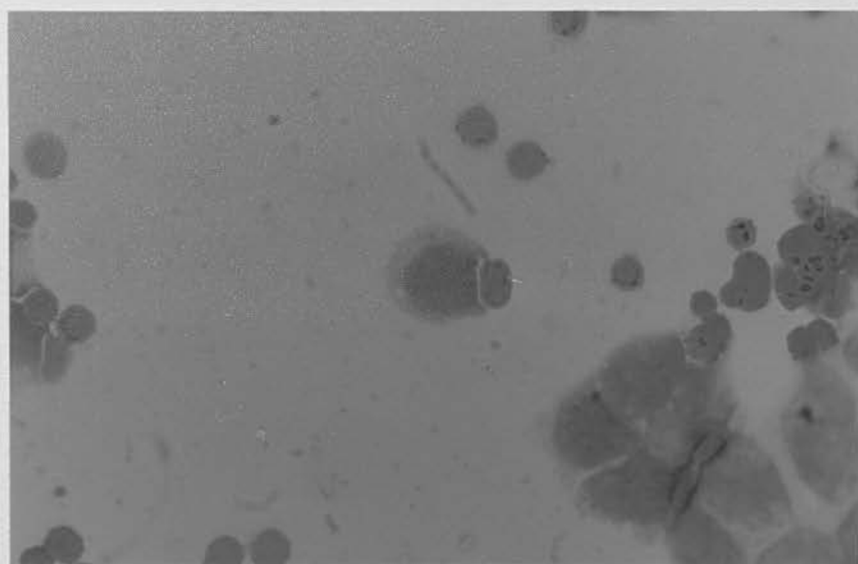
Further support for this in vivo is provided in Experiment 7.15 in which schizont particles from a volume of culture containing many calf-infective doses failed, on all occasions, to infect susceptible calves. The inference in this experiment is that the schizont particles can not sustain their viability or infectivity independent of the host cells.

Transmission of T. parva in vivo from the cells of male origin to female non-infected cells and vice versa was reported by Wilde et al (1966) who suggested a possible part played by macrophages in this. Brocklesby (1970) discussing the structural profile of extra-cellular schizonts in vivo indicated that, these could well be multinucleate merozoites and bodies responsible for the re-invasion of fresh lymphocytes in vivo. The conditions in the host animal are, however, different from those in vitro for transmission of schizonts from infected cells to non-infected ones. The difference can be explained as firstly, the macrophages in the animal are plentiful, genuine, autologous and active which they are not necessarily in vitro; secondly, theilerial bodies and schizont particles separated from the host cells might be subject to unnatural adverse conditions in vitro thus being incapable of maintaining viability in the extra-cellular form. Attempts to transfer schizonts from cell to cell in vitro, made by previous authors, with T. parva and T. annulata failed. Brocklesby (1956), in his first attempts to grow T. parva in tissue culture, drew attention to this point showing that the

parasite did not invade macrophages which were available in the cultures. Hulliger *et al* (1964) and Hulliger (1965) reported that *T. parva* and *T. annulata* never appeared in mitotic BHK cells which were used in the cultures as feeder layers. There are a number of reports indicating that schizonts of *T. parva* did not establish in bovine embryo spleen (BESP) cells, (Malmquist *et al* 1970; Moulton *et al* 1971a and 1971b; Malmquist and Brown 1974 and Stagg *et al* 1974).

Figs. 7.1 and 7.2

Phagocytosis of opsonised sheep red blood cells by normal cattle leucocytes.



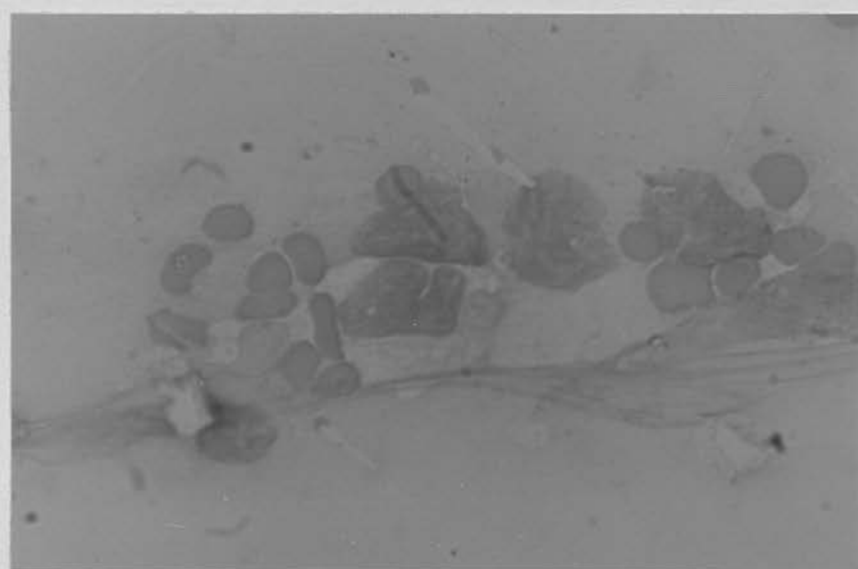
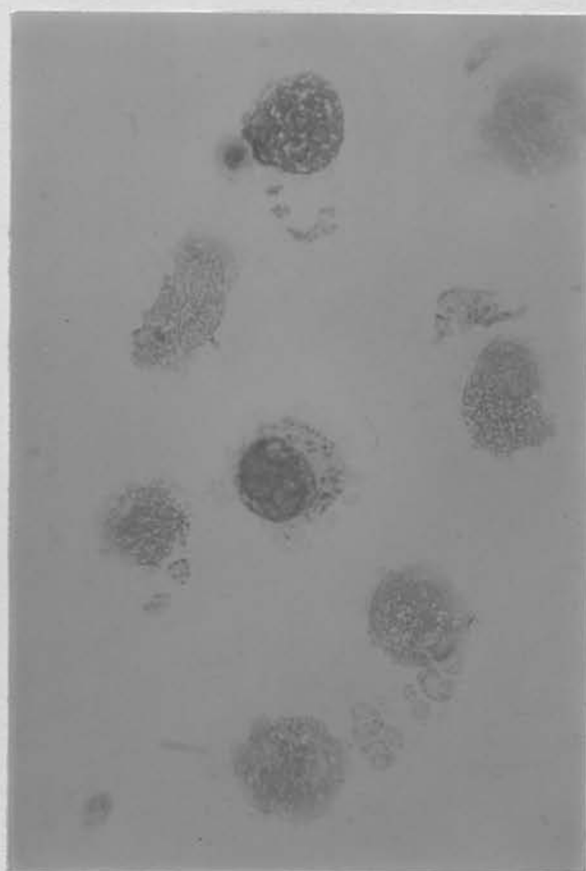


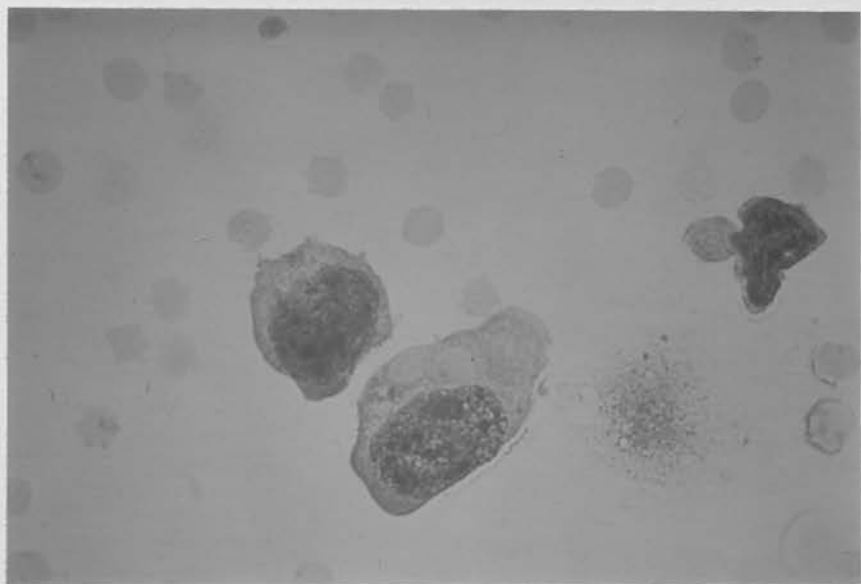
Fig. 7.3

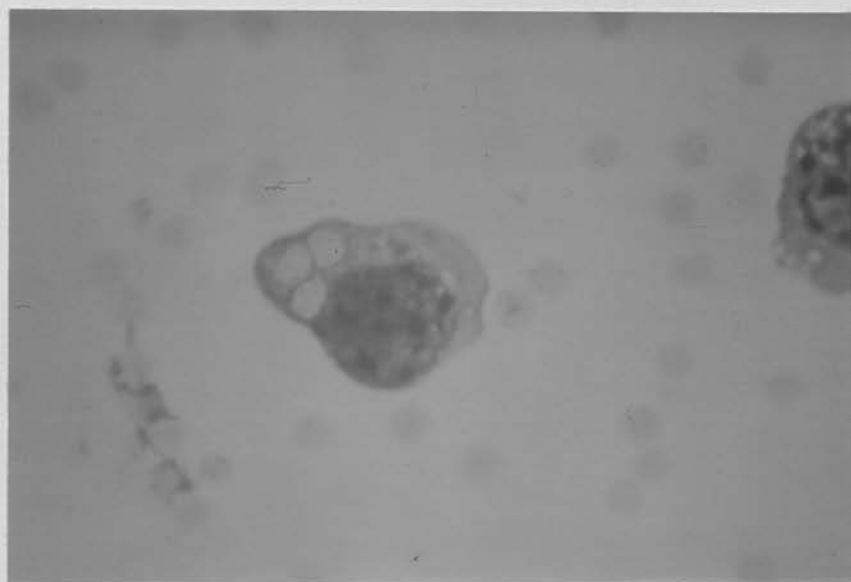
Free macroschizonts from suspension culture of schizont-infected cells.



Figs. 7.4, 7.5 and 7.6

Phagocytosis of opsonised sheep red blood cells by schizont-infected cells.





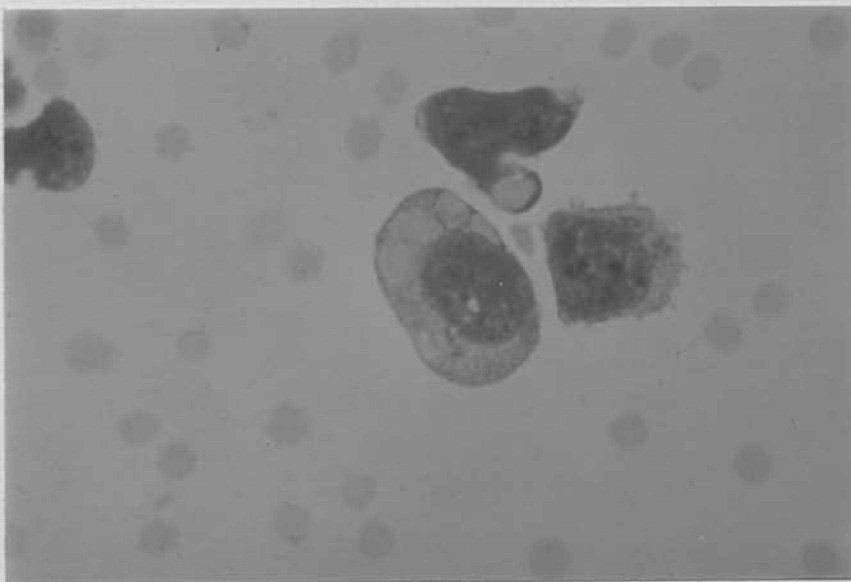
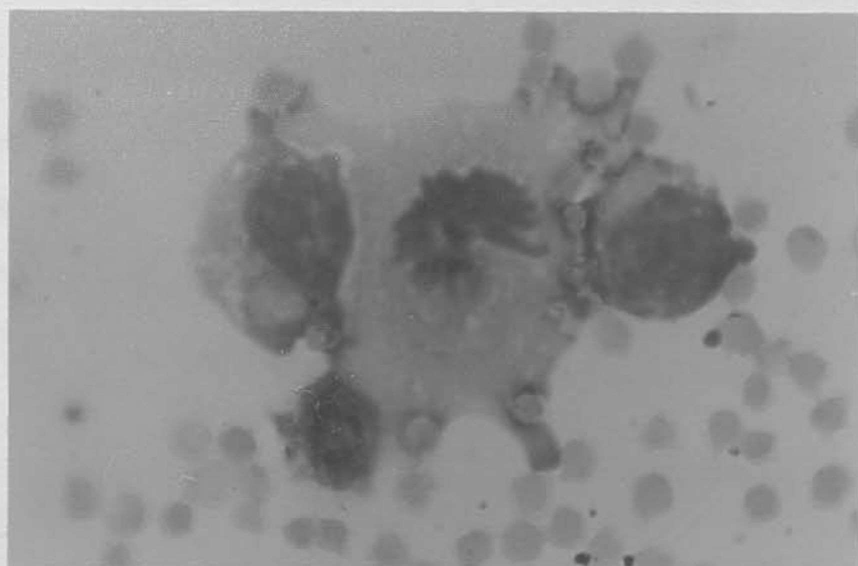


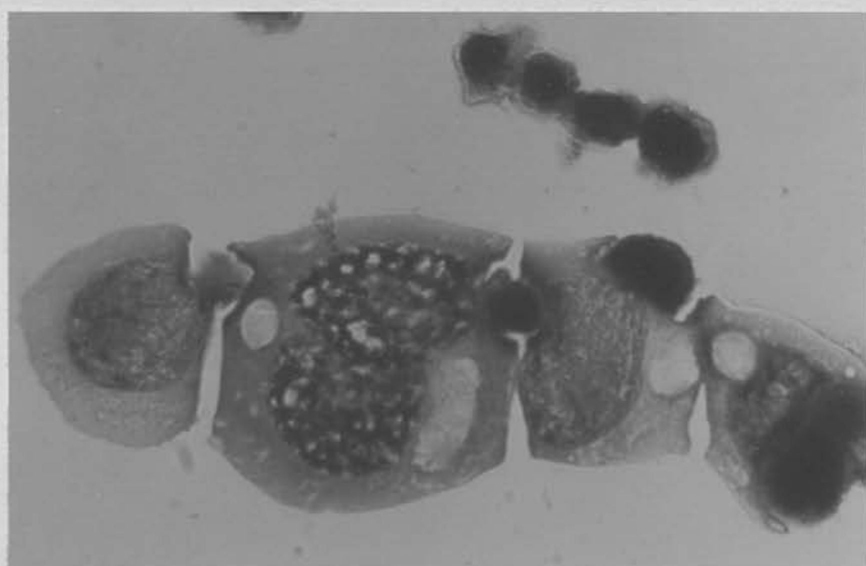
Fig. 7.7

Active phagocytosis of opsonised sheep red blood cells by a schizont-infected cell at mitosis.

Fig. 7.8

Phagocytosis of normal bovine lymphocytes by schizont-infected cells.





# CHAPTER 8

## GLUCOSE UPTAKE AND LACTATE PRODUCTION IN LYMPHOID CELLS INFECTED WITH <u>T. ANNULATA</u>

### A. Attempts to establish a normal uninfected cell line of cattle.

It appears that the schizonts of theilerial species are inseparable as viable entities from the host cells in which they reside. Any measurement of metabolism of the parasite in culture must, therefore, take into account the metabolic effect of the host cell itself. In an attempt to bring about a system in which reasonably accurate comparisons could be made, the following experiments were carried out: Experiments 8.1, 8.2 and 8.3 were designed to show whether or not active mitosis with subsequent successful suspension culture could be induced in non-infected bovine cells using phytohaemagglutinin (PHA) as a mitogen, so that a comparison of metabolism of such a culture could be made with cultures of infected cells which propagate successfully in suitable conditions (Experiment 8.4). Similar attempts were made using, as mitogen, staphylococcal filtrate (SF) and filtrate of theilerial infected cell cultures (Experiments 8.5 and 8.6). Healthy bovine lymphocytes have not so far been cultured without some means of stimulation. To make these normal cells undergo mitosis some mitogens such as PHA have been used by many authors in studies such as those concerning chromosome analysis (Nichols <u>et al</u> 1962; Biggers and McFeely 1963 and Ulbrich <u>et al</u> 1963). Therefore, the following experiments were carried out using some stimulants with the object of establishing a continuous suspension culture, even as a short

term control culture against schizont-infected cells in this study.

Experiment 8.1

Attempts to grow normal bovine lymphoid cells using PHA.

A lymph node from a normal calf was used as the source of cells which were separated as described in Chapter 3. The cells in suspension were shown to be 93.0 per cent viable. The medium used was Eagle's MEM with Earle's salts supplemented with ten per cent CS and 1.0g lactalbumin hydrolysate and 0.2g yeast extract (LY) per litre, respectively. The culture vessels used were 75cm$^2$ plastic Falcon flasks, 25cm$^2$ plastic T-flasks and ten ml plastic Leighton tubes. Numbers of viable cells were adjusted to 1.0 x 10$^6$ per ml of the final culture volume, as generally recommended. One series of the cultures received PHA (see Chapter 3) at the rate of 1.0ml reconstituted solution per 100ml v/v of medium. The other series of cultures was maintained without PHA as the control. The cultures were incubated at 37$^o$C and were sampled 24 hourly for cell counts and staining for five days. On the third day of cultivation samples from every culture, with and without PHA, were taken and used for chromosome preparations to confirm cell mitosis. The procedure was as described in Chapter 3. The cultures were then passaged every three to four days without further PHA in an attempt to obtain a continuous suspension culture.

RESULTS

In the stained smears of PHA treated cultures, very rare mitosis and blastoid cells were observed, while on the third day

when the chromosomes were prepared many mitoses were seen. The control cell culture did not show mitosis either in stained smears or in the chromosome preparations. This experiment was repeated several times with similar results. No continuous suspension culture was achieved either with or without PHA. The survival rates of the viable cells of these two cultures are shown in Table 8.1 and Fig. 8.1.

Table 8.1

The survival rates of the bovine lymphoid cells cultured with and without PHA.

| Days of incubation | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Culture with PHA | 100.0 | 80.0 | 72.0 | 72.5 | 60.0 | 30.5 |
| Control culture without PHA | 100.0 | 72.5 | 58.0 | 36.0 | 15.0 | 10.5 |

Number of viable cells per ml,x $10^4$

Experiment 8.2

Attempts to grow normal circulating bovine lymphocytes with PHA.

The lymphocytes were separated from heparinised blood of healthy cattle using gradient centrifugation as described in Chapter 3. The cell suspension was counted and adjusted to $1.0 \times 10^6$ cells per ml of medium. Viability of separated lymphocytes was tested and found to be 96.15 per cent. The cultures were set up with and without PHA as in the previous experiment. The samples were taken daily for five days for cell counts and staining. On the fourth day of cultivation samples

from both cultures were used for chromosome preparations to confirm the presence of mitosis. The cultures were then passaged every three to four days without PHA.

RESULTS

The cell culture including PHA showed blastoid cells and mitoses, both in stained smears and in preparations of the chromosomes. No mitosis was seen in either of the two preparations from the control culture. No mitosis nor growth were obtained in subsequent passages and the cells died off when PHA was not added. This experiment was repeated several times with the same results. The survival rate of the viable cells is shown in Table 8.2 and Fig. 8.2.

Table 8.2

The survival rate of bovine blood lymphocytes cultured with and without PHA.

| Days of incubation | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Culture with PHA | 100.0 | 87.5 | 80.5 | 77.5 | 60.5 | 42.5 |
| Culture without PHA | 100.0 | 75.0 | 50.0 | 37.5 | 22.0 | 15.0 |

Number of viable cells per ml, x $10^4$

Experiment 8.3

Attempts to grow normal bovine leucocytes with PHA.

Heparinised blood was prepared from a normal bovine animal. The leucocytes were separated from it using ammonium chloride, as described in Chapter 3. This cell suspension showed 90.9 per cent

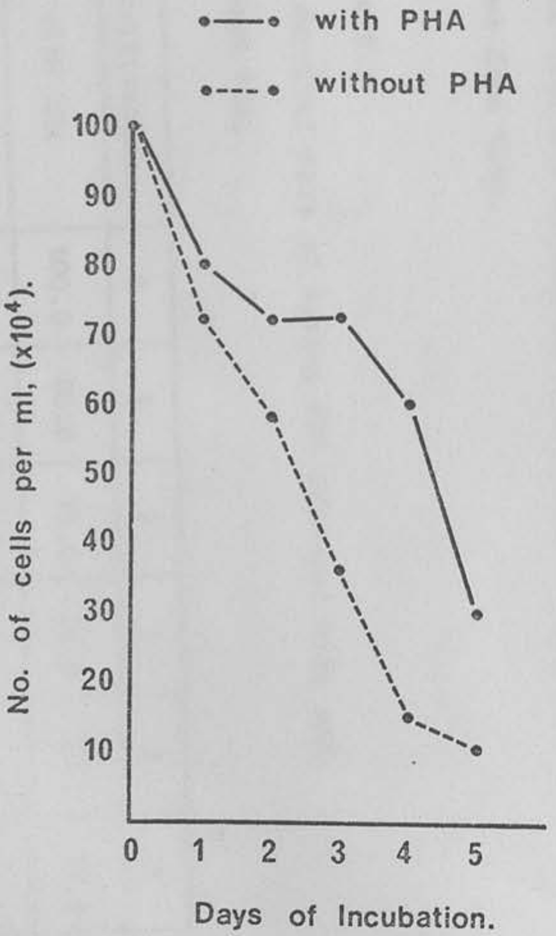



Fig. 8·1 Survival curves of viable lymphoid cells cultured with and without PHA.

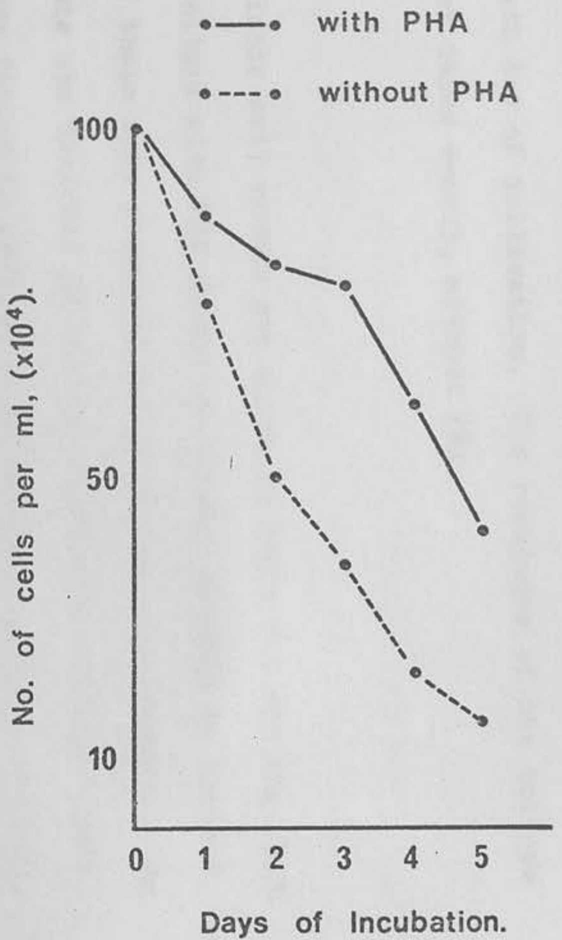



Fig. 8·2 Survival curves of viable blood lymphocytes cultured with and without PHA.

viability. The number of cells was adjusted to $1.0 \times 10^6$ cells per ml of medium. One series of the culture received PHA and the other was used as the control. These cultures were incubated and treated as in the previous experiments. Samples were taken daily for cell counts and staining. Chromosome preparations were made on the fourth day of cultivation. The remainder of the culture was passaged twice weekly, without PHA.

RESULTS

The viable cell counts are shown in Table 8.3 and Fig. 8.3. The cell culture with PHA showed occasional mitoses in stained smears and these were plentiful in chromosome preparations. No cell mitosis was observed in control suspension culture. Both preparations failed to produce a continuous suspension culture.

In all three experiments from five days onwards a number of adherent cells appeared attached to the surfaces of the culture vessels. These were versenated and brought to suspension at the appropriate time but continuous suspension culture was not established from them.

Table 8.3

The survival rate of bovine WBC cultured with and without PHA.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Culture with PHA | 100.0 | 80.0 | 75.0 | 70.0 | 55.0 | 37.5 |
| Culture without PHA | 100.0 | 60.0 | 50.5 | 40.0 | 22.5 | 10.0 |

Number of viable cells per ml, $\times 10^4$

Experiment 8.4

The effect of PHA on schizont-infected cells.

Since PHA appeared to cause some degree of multiplication of lymphocytes, although limited in amount and time, it was decided to see if PHA had any noticeable effect on cell multiplication in cultures of schizont-infected cells. In this experiment cultures of schizont-infected lymphoid cells were prepared containing 1.0 x $10^5$ viable cells per ml of medium. One series of the culture received one per cent v/v PHA, as in Experiment 8.1. This was the same amount as recommended for chromosome preparation by Nichols et al (1962) and the supplying manufacturers (Wellcome Reagents Limited). The control culture was maintained without PHA. These cultures were sampled 24 hourly for seven days for cell counts and morphological observation.

RESULTS

The stained smears from the PHA treated cultures showed the following abnormalities:-

(a) The schizont particles either inside the cells or in liberated theilerial bodies, appeared round, contracted and densely stained. Some of them were of small size, like micro-schizonts or transitional schizonts.

(b) Some vacuoles appeared in the cell cytoplasm.

(c) There were many dead cells. These cells were stained densely so that the schizonts were not discernible in them. Many of these cells were small and young infected cells.

(d) Large size and mature cells were disrupted releasing theilerial bodies and in some cases chromosomes. The cell counts are tabulated in Table 8.4 and Fig. 8.4.

Table 8.4

Growth rate of schizont-infected viable cells cultured with and without PHA.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Culture with PHA | 10.0 | 14.5 | 20.0 | 12.0 | 3.5 | 1.5 | 0.5 | 0.0 |
| Culture without PHA | 10.0 | 21.5 | 47.5 | 97.5 | 161.0 | 132.5 | 105.0 | 65.0 |

Number of viable cells per ml, x $10^4$

Experiment 8.5

Attempts to establish suspension cultures of lymphoid cells, blood lymphocytes and leucocytes from a normal bovine animal using a staphylococcal filtrate.

Staphylococcal filtrate (SF) has been proved to be a potent stimulant for lymphocytes of a number of animals (Knight, Ling, Sell and Oxnard 1965; Knight, Bradley, Oppenheim and Ling 1968; Ling and Kay 1975). This experiment was an attempt to determine whether or not a stimulant effect on (a) bovine lymphocytes from lymph nodes, (b) from the blood and (c) total blood leucocytes can be brought about by SF. Three sets of cultures of these cells (a), (b) and (c) were prepared, with 1.0 x $10^6$ viable cells per ml of medium in each, as described above. The medium used and other procedures of the cultivation were as described in Experiments 8.1, 8.2 and 8.3. The SF used was obtained from the Parasitology Laboratory of C.T.V.M. and was prepared according to Knight (1967). This was filtered through a 0.22µm millipore membrane and then added to the growth medium in this experiment to a final concentration of ten per cent, as recommended by Ling and Kay (1975).

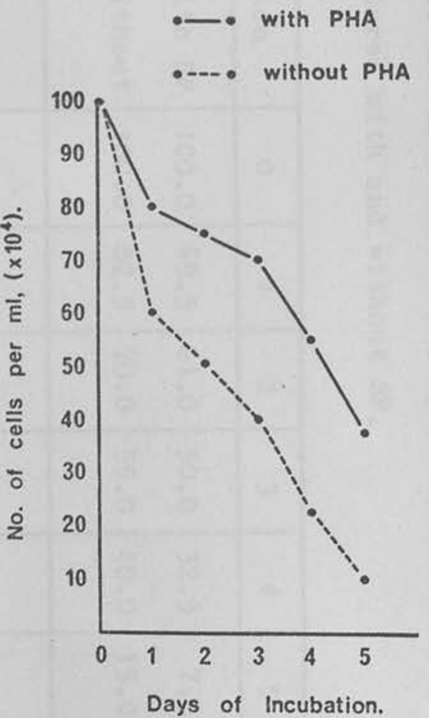



Fig. 8·3 Survival curves of viable WBC cultured with and without PHA.

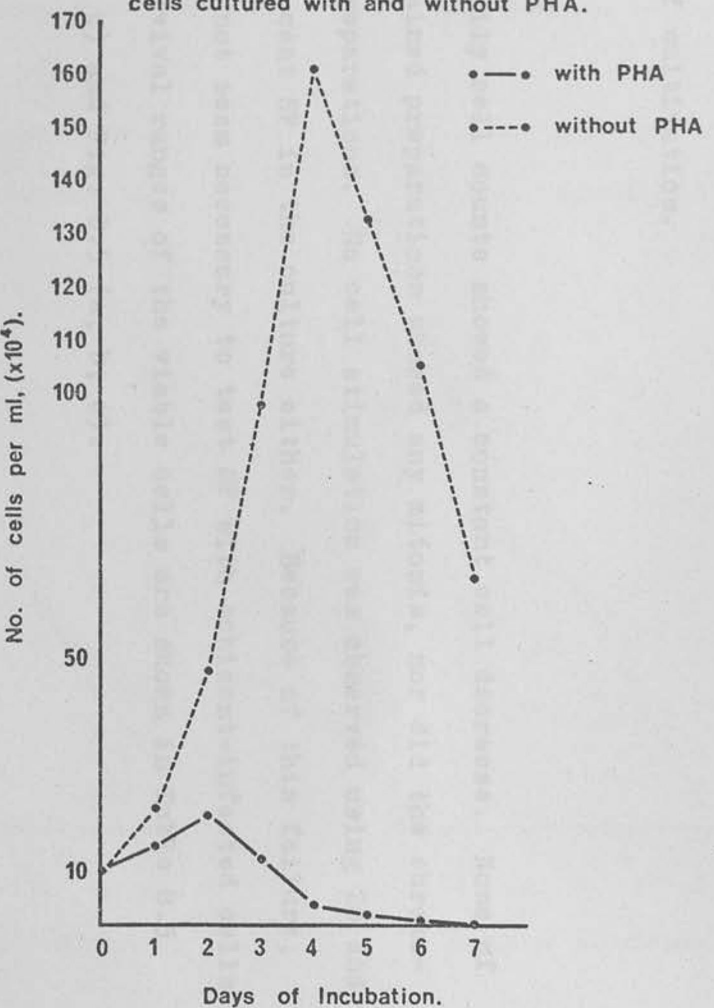



Fig. 8·4 Growth rate of schizont-infected viable cells cultured with and without PHA.

In later trials 20 and 30 per cent also were used. Daily cell counts and observations on stained smears were made for five days as well as chromosome preparations on the third and fourth days of cultivation.

RESULTS

Daily cell counts showed a constant cell decrease. None of the stained preparations showed any mitosis, nor did the chromosome preparations. No cell stimulation was observed using 20 and 30 per cent SF in the culture either. Because of this failure, it did not seem necessary to test SF with schizont-infected cells. The survival ranges of the viable cells are shown in Table 8.5 (a, b, c) and Fig. 8.5 (a, b, c).

Table 8.5

Survival rates of bovine lymphoid cells, lymphocytes and leucocytes cultured with and without SF.

| Trials | Days of incubation | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| a | Lymphoid cells with SF | 100.0 | 69.5 | 61.0 | 50.0 | 32.5 | 7.5 |
| | Lymphoid cells without SF | 100.0 | 82.5 | 70.0 | 55.0 | 40.0 | 15.0 |
| b | Blood lymphocytes with SF | 100.0 | 72.5 | 49.5 | 37.5 | 21.5 | 10.0 |
| | Blood lymphocytes without SF | 100.0 | 80.0 | 60.0 | 35.0 | 26.5 | 20.0 |
| c | WBC with SF | 100.0 | 67.5 | 45.0 | 40.0 | 21.5 | 5.0 |
| | WBC without SF | 100.0 | 70.0 | 55.0 | 47.5 | 25.0 | 12.5 |

Number of viable cells per ml, x $10^4$

Fig.8·5 (a) Survival curves of viable lymphoid cells with and without SF.

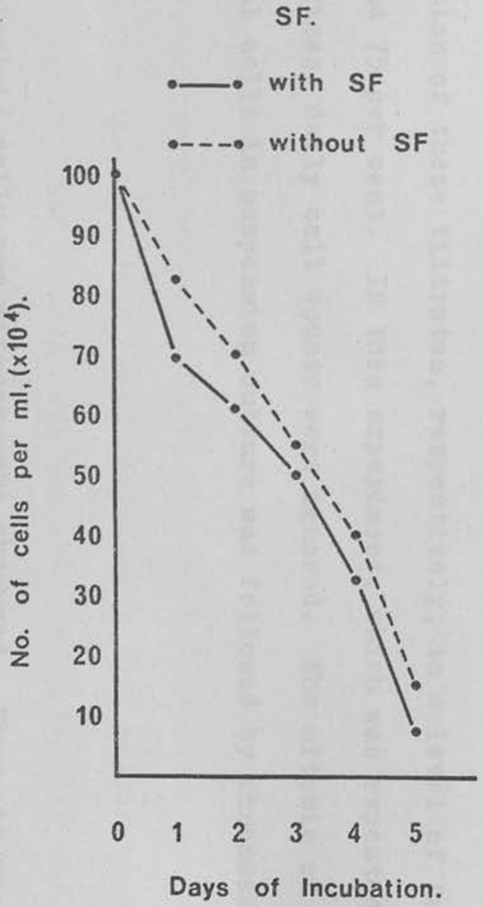



Fig. 8·5 (b) Survival curves of blood lymphocytes with and without SF.

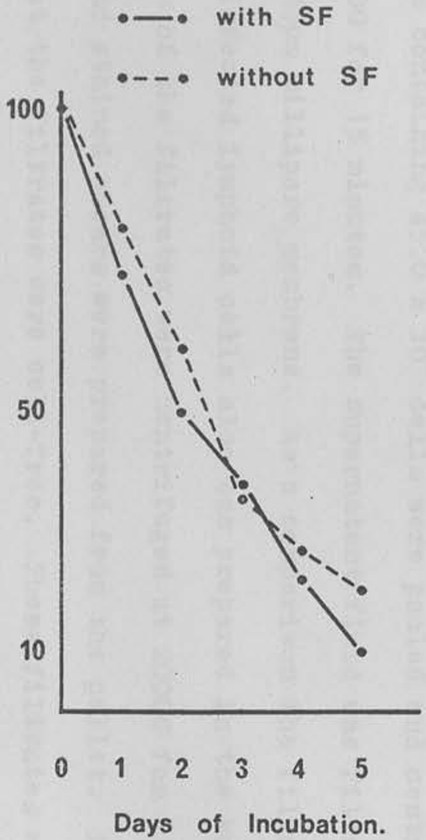
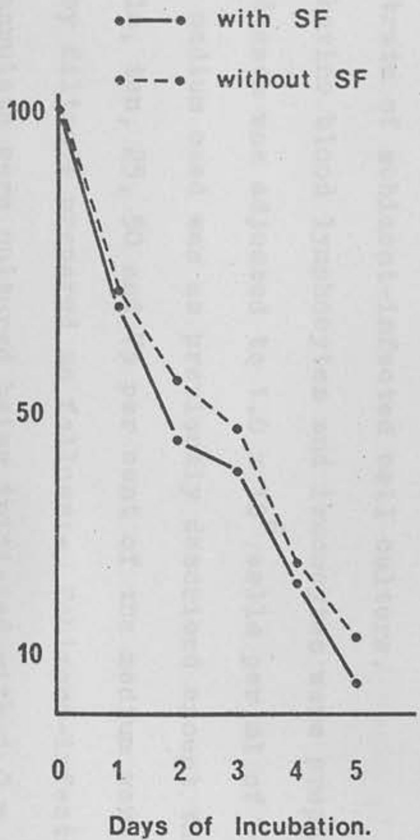



Fig. 8·5 (c) Survival curves of blood leucocytes with and without SF.



Experiment 8.6

Attempts to stimulate normal bovine blood cells using the filtrate of schizont-infected cell culture.

Normal bovine blood lymphocytes and leucocytes were prepared as before and each was adjusted to $1.0 \times 10^6$ cells per ml of medium. The medium used was as previously described except that in four trials, ten, 25, 50 and 75 per cent of the medium were substituted by filtrate prepared as follows:- Schizont-infected cells of *T. annulata* were cultured being initiated with $1.0 \times 10^5$ cells per ml of medium and incubated for four days. Thirty ml cell cultures containing $45.0 \times 10^6$ cells were pooled and centrifuged at 2000G for 15 minutes. The supernatant fluid was filtered through a 0.45µm millipore membrane. As a comparison the filtrate of *T. parva*-infected lymphoid cells also was prepared in the same way. Samples of the filtrates were centrifuged at 2000G for 30 minutes and stained smears were prepared from the pellet. It was shown that the filtrates were cell-free. These filtrates were dispensed in Universal bottles and were kept at -24°C until used. The normal cells were suspended in the medium as before with the substitution of these filtrates, respectively, to a level of ten, 25, 50 and 75 per cent. In this experiment, which was repeated several times, daily cell counts were ignored. The mitosis of the normal cells in suspension culture was followed by chromosome analysis.

RESULTS

No blastoid cells nor mitoses were observed. Thus, it was found that these filtrates could not stimulate the blood cells to undergo mitosis.

B. Glucose uptake and lactate production.

General procedure of the experiments.

Since the early work on tissue culture, the importance of carbohydrate as a constituent of the media used has been well demonstrated. The most commonly available source of carbohydrate is glucose (Roos and Loos 1970; Paul 1975 and others). The amount of glucose required varies in normal and malignant cells (Paul 1975). In synthetic growth media the amount of glucose can be varied. To understand how much of the glucose is utilised by <u>T. annulata</u>-infected cells, the following experiments were carried out. The estimation of glucose consumption and lactate production was made in relation to growth rate of the infected cells over various periods of time. <u>Theileria</u>-infected cells were set up in cultures at a level of 1.0 x $10^5$ viable cells per ml of medium. The medium used was Eagle's MEM with Earle's salts plus ten per cent CS and supplemented with LY and NEAA (see Chapter 3). The pH of the medium was adjusted to 7.0 at 37°C using Hepes buffer and sodium bicarbonate. In one experiment, 8.8, one extra gramme of glucose was added to the medium. The initial medium, with 1.0g glucose per litre was referred to as growth medium No. I and that containing 2.0g glucose per litre was designated growth medium No. II, for simplicity. The cell cultures were incubated at 37°C except in Experiment 8.10 which was carried out at various temperatures. The culture vessels used were ten ml plastic Leighton tubes. Procedures for the viability test, cell counts and preparation of stained smears from the cell culture which were carried out daily, were as described in Chapter 3.

Comparisons of growth rate with uptake of glucose and production of lactate were made using the following systems:-

(i) Schizont-infected cells, S.15 in growth media Nos. I and II i.e., containing 1.0g and 2.0g glucose per litre, respectively at 37°C.

(ii) Schizont-infected cells, S.15 and S.3, respectively, in medium No. I at 37°C.

(iii) Schizont-infected cells, S.15 in medium No. I at temperatures, 37°C, 25°C, 35°C, 39°C and 40°C, respectively.

(iv) <u>T. parva</u>-infected cells in medium No. I at 37°C.

(v) In addition, as control, medium No. I without cells was incubated at 37°C and examined for variations in glucose and lactate content (Experiment 8.7).

Experiment 8.7

Control measurement of glucose and lactate from growth medium and supplements.

Before the experiments with cultured cells the following measurements were carried out:-

(a) Samples from growth medium No. I, from growth medium No. II, from Eagle's MEM without any supplement, from CS and from stock solution of LY were prepared for glucose estimation. This was without any incubation.

(b) Samples from only medium No. I for glucose estimation and (c) from this medium for lactate estimation both were prepared daily during Day 0 to Day 9 of incubation. The purpose was to differentiate quantitative changes of glucose and lactate caused by incubation alone. Three ml aliquots of the growth medium No. I were dispensed in each of ten plastic Leighton tubes without any cells. These were numbered from zero to nine, screwed tightly and incubated at 37°C. The tube zero was taken after an hour

when the medium was warmed up and samples for glucose and lactate measurements were prepared as Day 0 samples. The other tubes were treated, similarly 24 hourly, sequentially. The samples were deproteinised as described in Chapter 3 and were stored at -24°C until used.

RESULTS

(a) The amounts of glucose measured from the media and supplements are shown in Table 8.6(a).

(b) Glucose values measured from the medium No. I, incubated at 37°C from Day 0 to Day 9 are demonstrated in Table 8.6(b) and Fig.8.6

(c) Lactate values measured from the medium No. I, incubated at 37°C from Day 0 to Day 9 are shown in Table 8.6(c) and in Fig. 8.6.

Table 8.6(a)

Glucose values measured from the medium No. I, the medium No. II, non-supplemented Eagle's MEM, CS and LY.

| Media and supplements | Medium No. I | Medium No. II | Eagle's MEM | CS | LY |
|---|---|---|---|---|---|
| O.D. Samples | 0.240 | 0.458 | 0.218 | 0.215 | 0.000 |
| Glucose/litre (mg) | 1090.9 | 2081.8 | 990.9 | 977.3 | 0.000 |

Optical density (O.D.) of the Standard = 0.220

The above values are means of two measurements.

Calculation: $\frac{\text{O.D. Sample}}{\text{O.D. Standard}} \times 1000 =$ mg glucose/litre

Table 8.6(b)

Glucose values measured from growth medium No. I, incubated at 37°C from Day 0 to Day 9.

| Days | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| O.D. Samples | 0.239 | 0.240 | 0.238 | 0.238 | 0.234 | 0.232 | 0.230 | 0.230 | 0.228 | 0.229 |
| Glucose/litre (mg) | 1086.4 | 1090.9 | 1081.8 | 1081.8 | 1063.6 | 1054.5 | 1045.5 | 1045.5 | 1036.4 | 1040.9 |

O.D. Standard = 0.220

The above values are means of two measurements

Table 8.6(c)

Lactate values measured from growth medium No. I, incubated at 37°C from Day 0 to Day 9

| Days | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| O.D. Samples (Es) | 0.738 | 0.741 | 0.747 | 0.745 | 0.758 | 0.762 | 0.768 | 0.773 | 0.773 | 0.780 |
| Lactate/litre (mg) | 167.7 | 169.2 | 172.2 | 171.2 | 177.6 | 179.6 | 182.6 | 185.1 | 185.1 | 188.6 |

The above values are means of two measurements

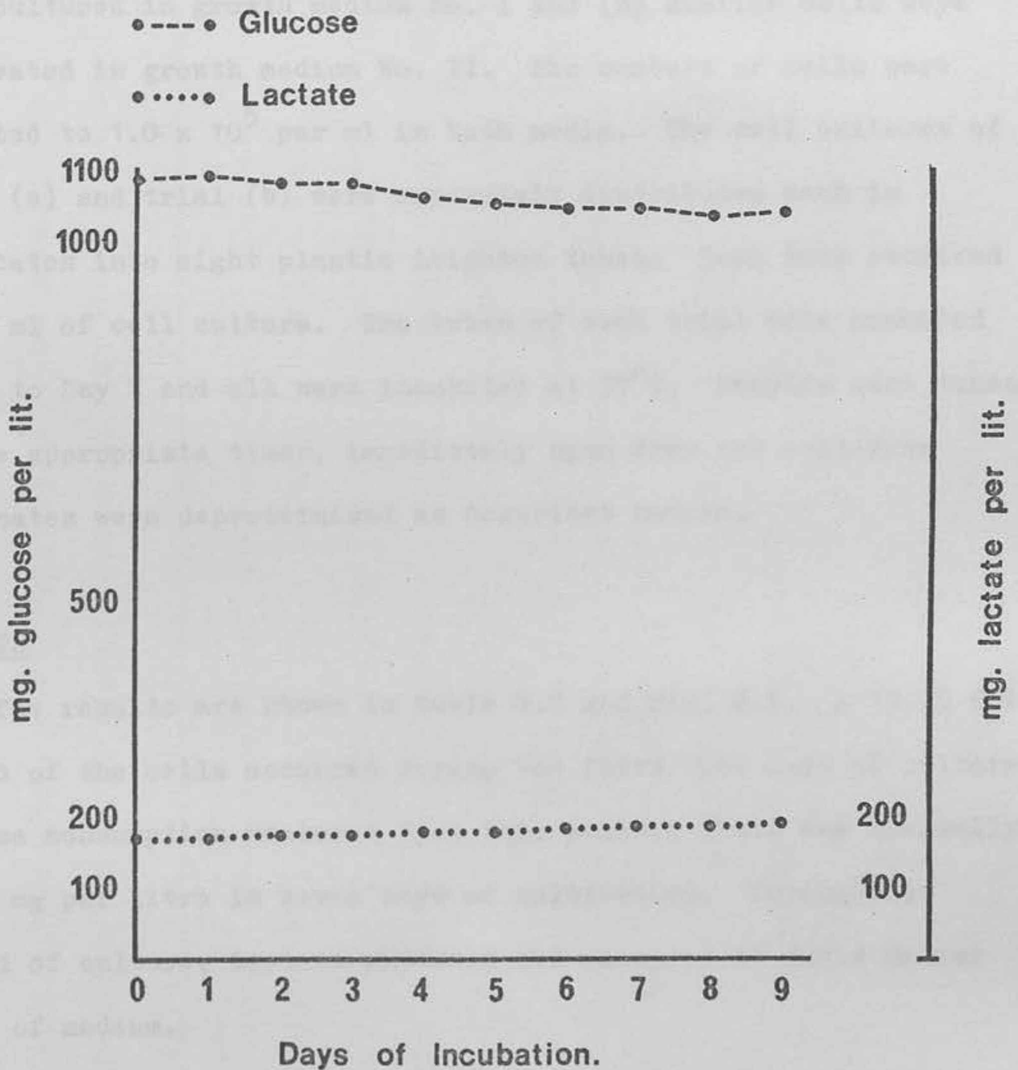
Es = optical density of the sample

$E_b$ = " " " " blank = 0.402

Calculation : $Es - E_b = \Delta E$

Concentration : $c = 499 \times \Delta E_{340nm}$ (mg/1000ml) $\triangle$

Fig. 8·6 Glucose and lactate values measured from growth medium No. I, incubated at 37°C from Day 0 to Day 9.



Experiment 8.8

Glucose uptake and lactate production by schizont-infected cells in the growth media Nos. I and II.

To determine whether or not the amount of glucose in growth medium affects the glucose uptake and lactate production, two trials were carried out. (a) *T. annulata*-infected cells (S.15) were cultured in growth medium No. I and (b) similar cells were cultivated in growth medium No. II. The numbers of cells were adjusted to $1.0 \times 10^5$ per ml in both media. The cell cultures of trial (a) and trial (b) were separately distributed each in duplicates into eight plastic Leighton tubes. Each tube received three ml of cell culture. The tubes of each trial were numbered Day 0 to Day 7 and all were incubated at $37^{\circ}C$. Samples were taken at the appropriate times, immediately spun down and cell-free supernates were deproteinised as described before.

RESULTS

(a) The results are shown in Table 8.7 and Fig. 8.7. A 19.75 fold growth of the cells occurred during the first five days of culture. Glucose consumption measured from this culture fluid was maximally 801.9 mg per litre in seven days of cultivation. During this period of culture, lactate produced was measured as 284.4 mg per litre of medium.

(b) The results are shown in Table 8.8 and Fig. 8.8. Schizont-infected cells showed a 19.25 fold increase in the first five days. In this growth medium No. II, 754.7 mg glucose were used in seven days of culture and lactate was increased up to 240.5 mg per litre of medium over the same period.

Table 8.7

Glucose uptake and lactate production in relation to growth rate of

<u>T. annulata</u>-infected cells (S.15) grown in medium No. I.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 26.5 | 47.5 | 88.5 | 148.0 | 197.5 | 165.5 | 125.5 |
| O.D. samples | 0.232 | 0.222 | 0.204 | 0.165 | 0.113 | 0.075 | 0.067 | 0.062 |
| Glucose/litre (mg) | 1094.3 | 1047.2 | 962.3 | 778.3 | 533.0 | 353.8 | 316.0 | 292.4 |
| O.D. samples (Es) | 0.770 | 0.812 | 0.985 | 1.044 | 1.228 | 1.300 | 1.262 | 1.340 |
| Lactate/litre (mg) | 183.6 | 204.6 | 290.9 | 320.4 | 412.2 | 448.1 | 429.1 | 468.0 |

In glucose measurement, O.D. Standard = 0.212

In lactate " O.D. of blank or $E_b$ = 0.402

The above values are all means of two cultures.

Fig. 8·7 Glucose uptake, lactate production in relation to growth rate of S.15, *T. annulata*-infected cells in growth medium No. I.

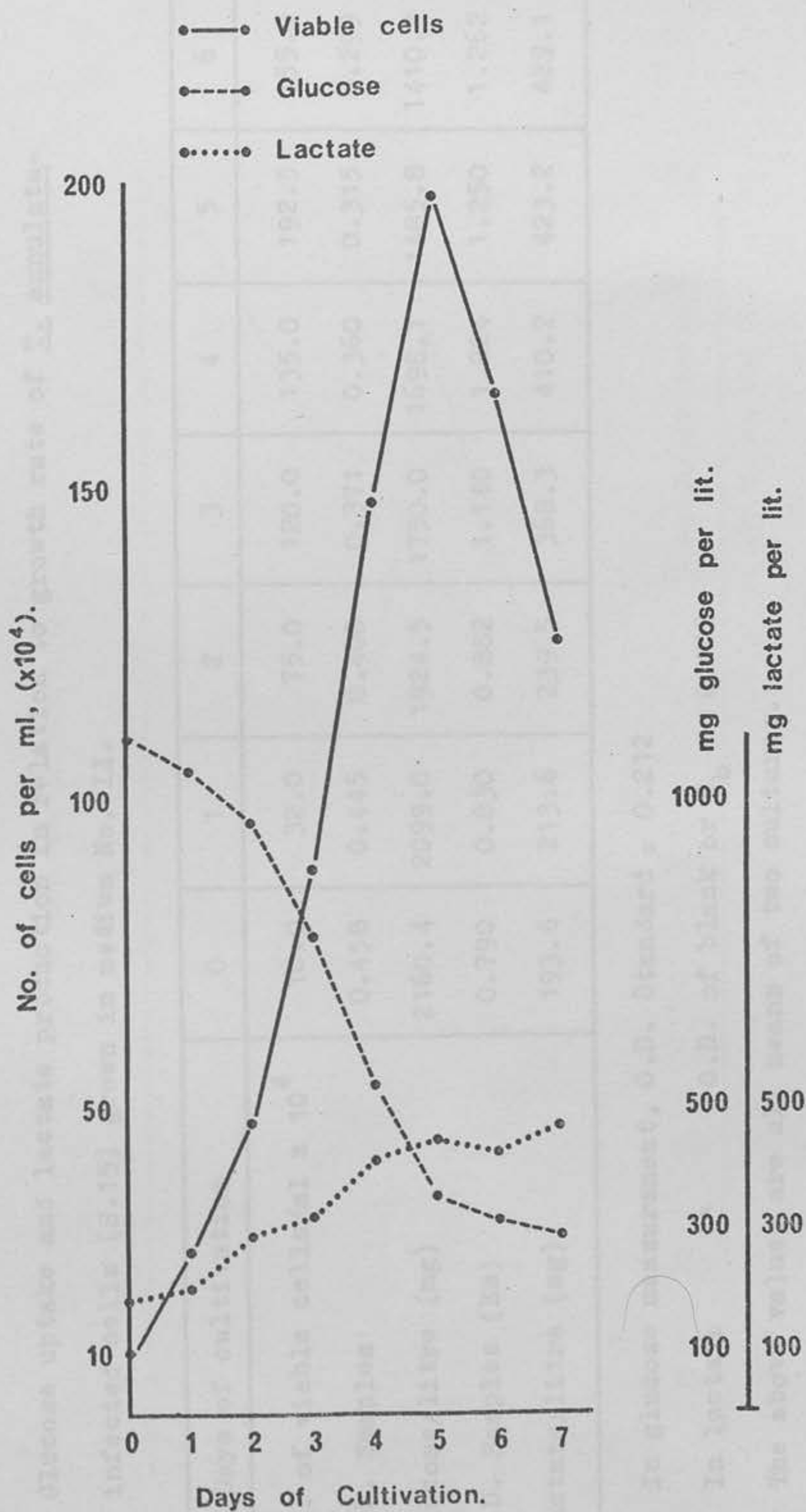


Table 8.8

Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells (S.15) grown in medium No. II.

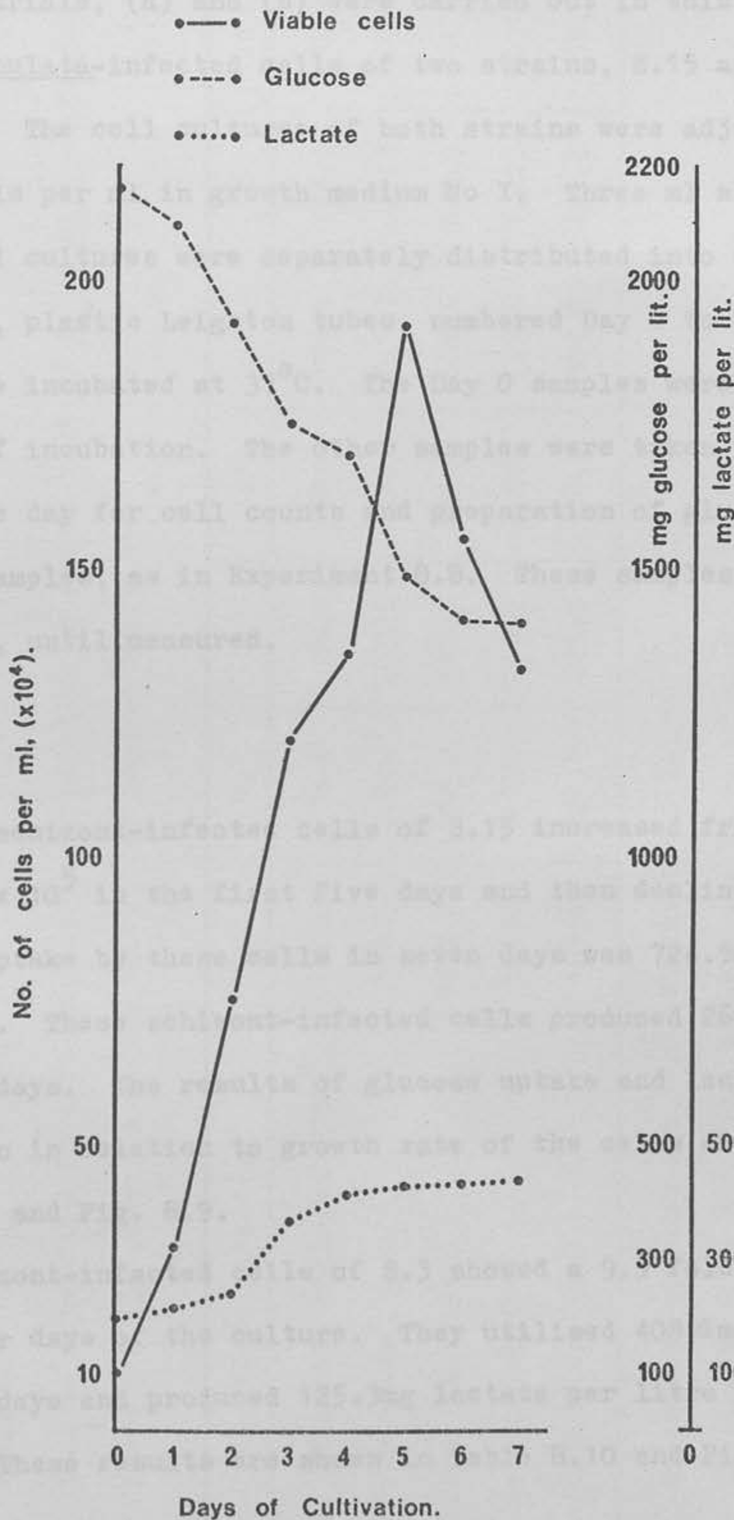
| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 32.0 | 75.0 | 120.0 | 135.0 | 192.5 | 155.0 | 132.5 |
| O.D. Samples | 0.458 | 0.445 | 0.408 | 0.371 | 0.360 | 0.315 | 0.299 | 0.298 |
| Glucose/litre (mg) | 2160.4 | 2099.0 | 1924.5 | 1750.0 | 1698.1 | 1485.8 | 1410.4 | 1405.7 |
| O.D. Samples (Es) | 0.790 | 0.830 | 0.882 | 1.140 | 1.224 | 1.250 | 1.262 | 1.272 |
| Lactate/litre (mg) | 193.6 | 213.6 | 239.5 | 368.3 | 410.2 | 423.2 | 429.1 | 434.1 |

In glucose measurement, O.D. Standard = 0.212

In lactate " O.D. of blank or $E_b$ = 0.402

The above values are all means of two cultures.

Fig. 8·8 Glucose uptake and lactate production in relation to growth rate of S.15, T. annulata-infected cells in growth medium No.II.



Experiment 8.9

Glucose uptake and lactate production by T. annulata-infected cells (S.15) and (S.3) grown in medium No. I.

Two trials, (a) and (b) were carried out in this experiment and T. annulata-infected cells of two strains, S.15 and S.3 were compared. The cell cultures of both strains were adjusted to 1.0 x $10^5$ cells per ml in growth medium No I. Three ml aliquots of these cell cultures were separately distributed into eight duplicate, plastic Leighton tubes, numbered Day 0 to Day 7. The tubes were incubated at 37°C. The Day 0 samples were prepared after an hour of incubation. The other samples were taken every successive day for cell counts and preparation of glucose and lactate samples, as in Experiment 8.8. These samples were stored at -24°C, until measured.

RESULTS

(a) The schizont-infected cells of S.15 increased from 1.0 x $10^5$ to 18.85 x $10^5$ in the first five days and then declined. The glucose uptake by these cells in seven days was 724.5mg per litre of medium. These schizont-infected cells produced 269.5mg lactate in seven days. The results of glucose uptake and lactate production in relation to growth rate of the cells are shown in Table 8.9 and Fig. 8.9.

(b) Schizont-infected cells of S.3 showed a 9.5 fold rise in the first four days of the culture. They utilised 408.8mg of glucose in seven days and produced 125.3mg lactate per litre in the same period. These results are shown in Table 8.10 and Fig. 8.10.

Table 8.9

Glucose uptake and lactate production in relation to growth rate of *T. annulata*-infected cells (S.15) grown in medium No. I, (as control for S.3)

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 18.5 | 35.0 | 72.0 | 165.0 | 188.5 | 152.5 | 110.0 |
| O.D. samples | 0.245 | 0.248 | 0.220 | 0.187 | 0.137 | 0.117 | 0.088 | 0.082 |
| Glucose/litre (mg) | 1088.9 | 1102.2 | 977.8 | 831.1 | 608.9 | 520.0 | 391.1 | 364.4 |
| O.D. samples (Es) | 0.785 | 0.808 | 0.865 | 1.015 | 1.156 | 1.270 | 1.310 | 1.325 |
| Lactate/litre (mg) | 189.6 | 201.1 | 229.5 | 304.4 | 374.7 | 431.6 | 451.6 | 459.1 |

In glucose measurement, O.D. Standard = 0.225

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures.

Fig. 8·9 Glucose uptake and lactate production in relation to growth rate of T. annulata - infected cells, S.15 grown in medium No.I (as control for S.3).

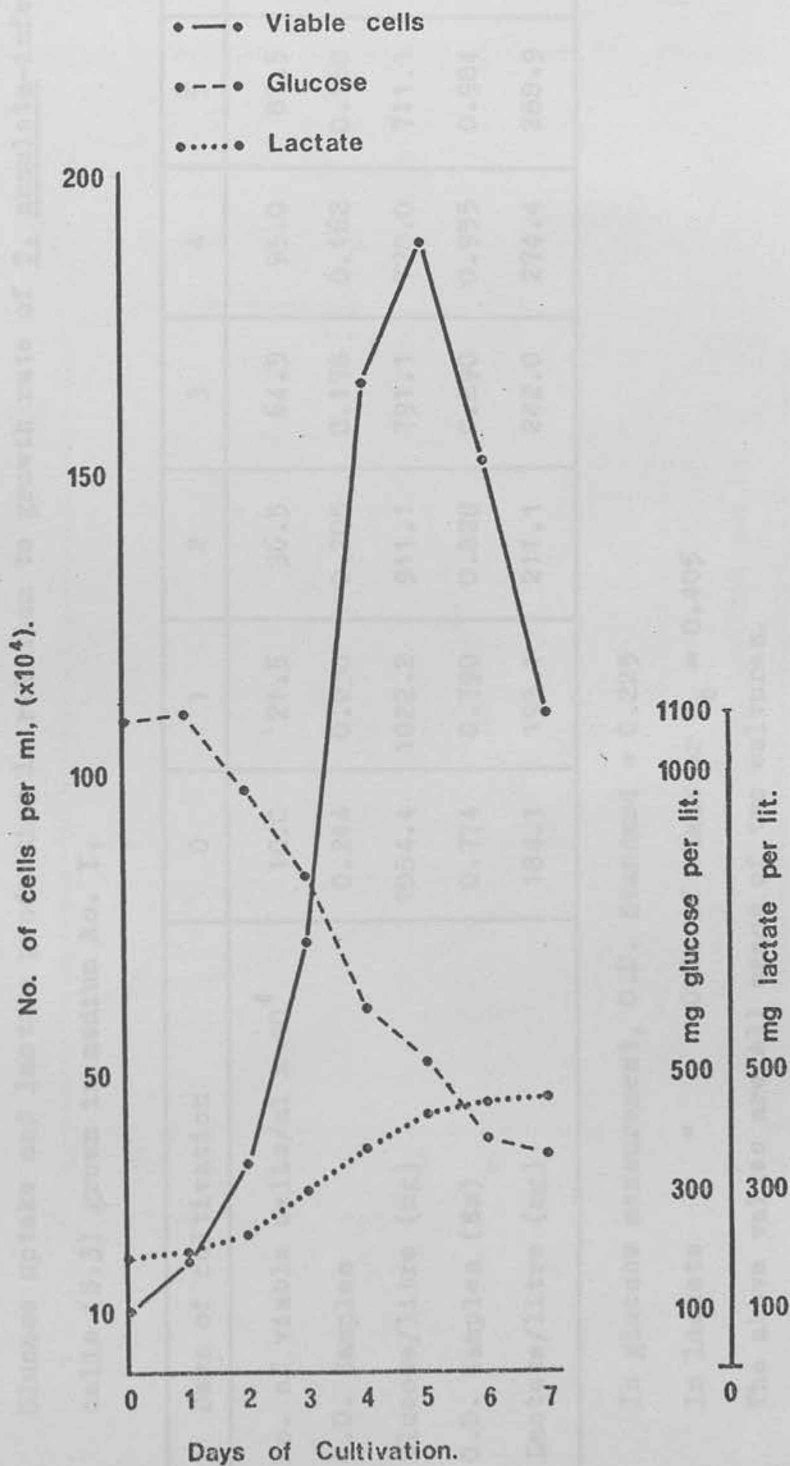


Table 8.10

Glucose uptake and lactate production in relation to growth rate of *T. annulata*-infected cells (S.3) grown in medium No. I.

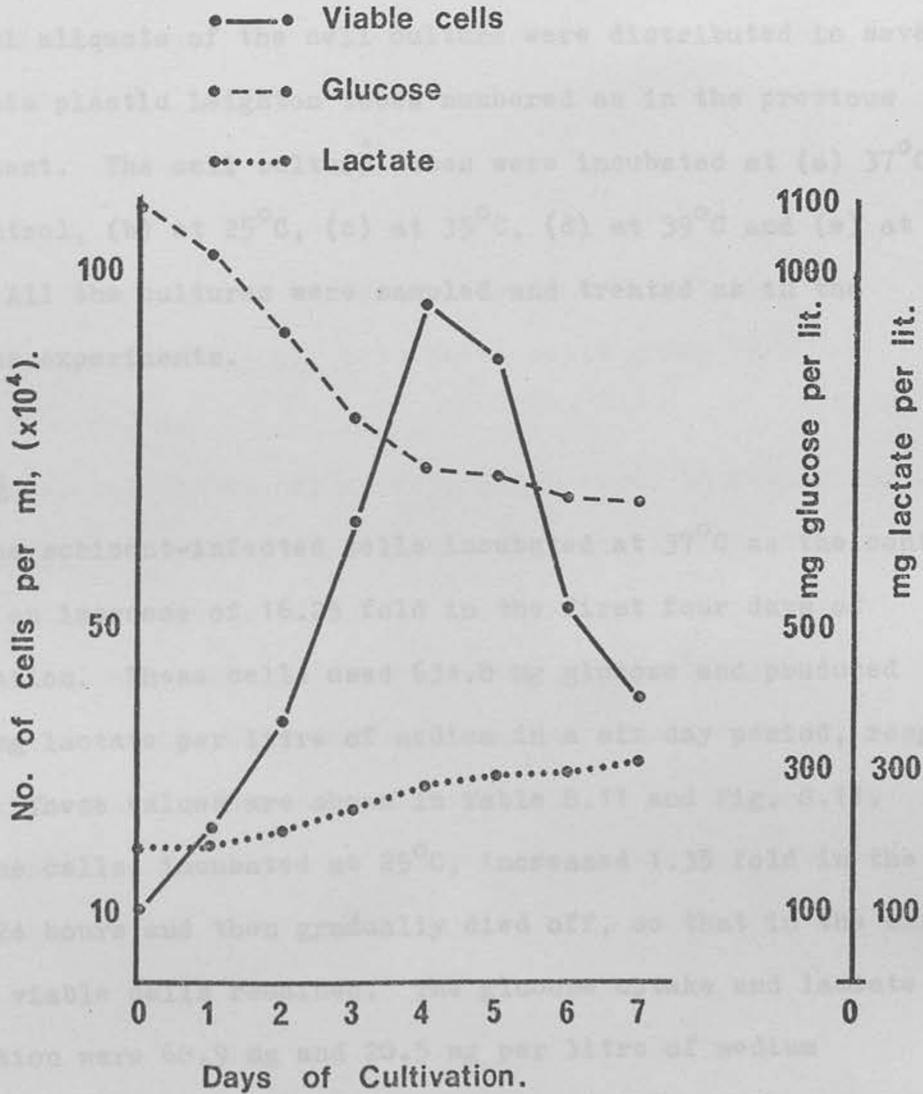
| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 21.5 | 36.5 | 64.5 | 95.0 | 87.5 | 52.5 | 40.0 |
| O.D. Samples | 0.244 | 0.230 | 0.205 | 0.178 | 0.162 | 0.160 | 0.153 | 0.152 |
| Glucose/litre (mg) | 1084.4 | 1022.2 | 911.1 | 791.1 | 720.0 | 711.1 | 680.0 | 675.6 |
| O.D. Samples (Es) | 0.774 | 0.790 | 0.828 | 0.890 | 0.955 | 0.984 | 1.000 | 1.025 |
| Lactate/litre (mg) | 184.1 | 192.1 | 211.1 | 242.0 | 274.4 | 288.9 | 296.9 | 309.4 |

In glucose measurement, O.D. Standard = 0.225

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures.

Fig. 8·10 Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells, S.3 grown in medium No. I.



Experiment 8.10

Glucose uptake and lactate production by T. annulata-infected cells (S.15) grown in medium No. I at various temperatures.

(e) In this experiment schizont-infected cells, S.15 were used at 1.0 x $10^5$ cells per ml in growth medium No. I. Five trials at five different temperatures were carried out. In each trial three ml aliquots of the cell culture were distributed in seven duplicate plastic Leighton tubes numbered as in the previous experiment. The cell culture tubes were incubated at (a) 37°C as the control, (b) at 25°C, (c) at 35°C, (d) at 39°C and (e) at 40°C. All the cultures were sampled and treated as in the previous experiments.

RESULTS

(a) The schizont-infected cells incubated at 37°C as the control showed an increase of 16.25 fold in the first four days of cultivation. These cells used 634.8 mg glucose and produced 230.6 mg lactate per litre of medium in a six day period, respectively. These values are shown in Table 8.11 and Fig. 8.11.

(b) The cells, incubated at 25°C, increased 1.35 fold in the first 24 hours and then gradually died off, so that in the sixth day no viable cells remained. The glucose uptake and lactate production were 60.9 mg and 20.5 mg per litre of medium respectively, as shown in Table 8.12 and Fig. 8.12.

(c) At 35°C the viable cells increased 5.5 fold in the first four days. The glucose uptake was 226.0 mg per litre and the lactate produced was 79.9 mg per litre of medium. The values are shown in Table 8.13 and Fig. 8.13.

(d) The cells incubated at 39°C, showed 9.75 fold growth in five days. These cells utilised 413.0 mg glucose and produced 140.7 mg lactate per litre of medium in six days, (Table 8.14 and Fig. 8.14).

(e) The growth rate of viable schizont-infected cells at 40°C reached 2.4 fold in the first three days then declined. Glucose was utilised up to 108.7 mg and lactate was produced up to 44.4 mg per litre of medium. The above values are shown in Table 8.15 and Fig. 8.15.

Experiment 8.11

Glucose uptake and lactate production in relation to growth rate of T. parva-infected cells grown in medium No. I.

Schizont-infected cells of T. parva were, in comparison with T. annulata, cultivated in growth medium No. I. The number of viable cells was adjusted to 1.0 x $10^5$ per ml and the cell culture was then distributed into eight duplicate plastic Leighton tubes, numbered Day 0 to Day 7. Each tube received three ml of the cell culture. They were incubated at 37°C and the samples were prepared at appropriate times as described in previous experiments.

RESULTS

The growth rate of schizont-infected cells increased 4.8 fold in the first four days. Glucose was used up to 217.8 mg and lactate was produced up to 59.9 mg per litre of medium. The above values are shown in Table 8.16 and Fig. 8.16.

Table 8.11

Glucose uptake and lactate production in relation to growth rate of *T. annulata*-infected cells (S.15) grown in medium No. I, at 37° C (the control).

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 17.5 | 37.5 | 75.0 | 162.5 | 152.5 | 125.0 |
| O.D. Samples | 0.248 | 0.240 | 0.216 | 0.185 | 0.135 | 0.120 | 0.102 |
| Glucose/litre (mg) | 1078.3 | 1043.5 | 939.1 | 804.3 | 586.9 | 521.7 | 443.5 |
| O.D. Samples (Es) | 0.758 | 0.785 | 0.845 | 0.955 | 1.140 | 1.186 | 1.220 |
| Lactate/litre (mg) | 176.1 | 189.6 | 219.6 | 274.4 | 366.8 | 389.7 | 406.7 |

In glucose measurement, O.D. Standard = 0.230

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures

Fig.8·11 Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells, S. 15 grown in medium No.I (at 37°C, the control).

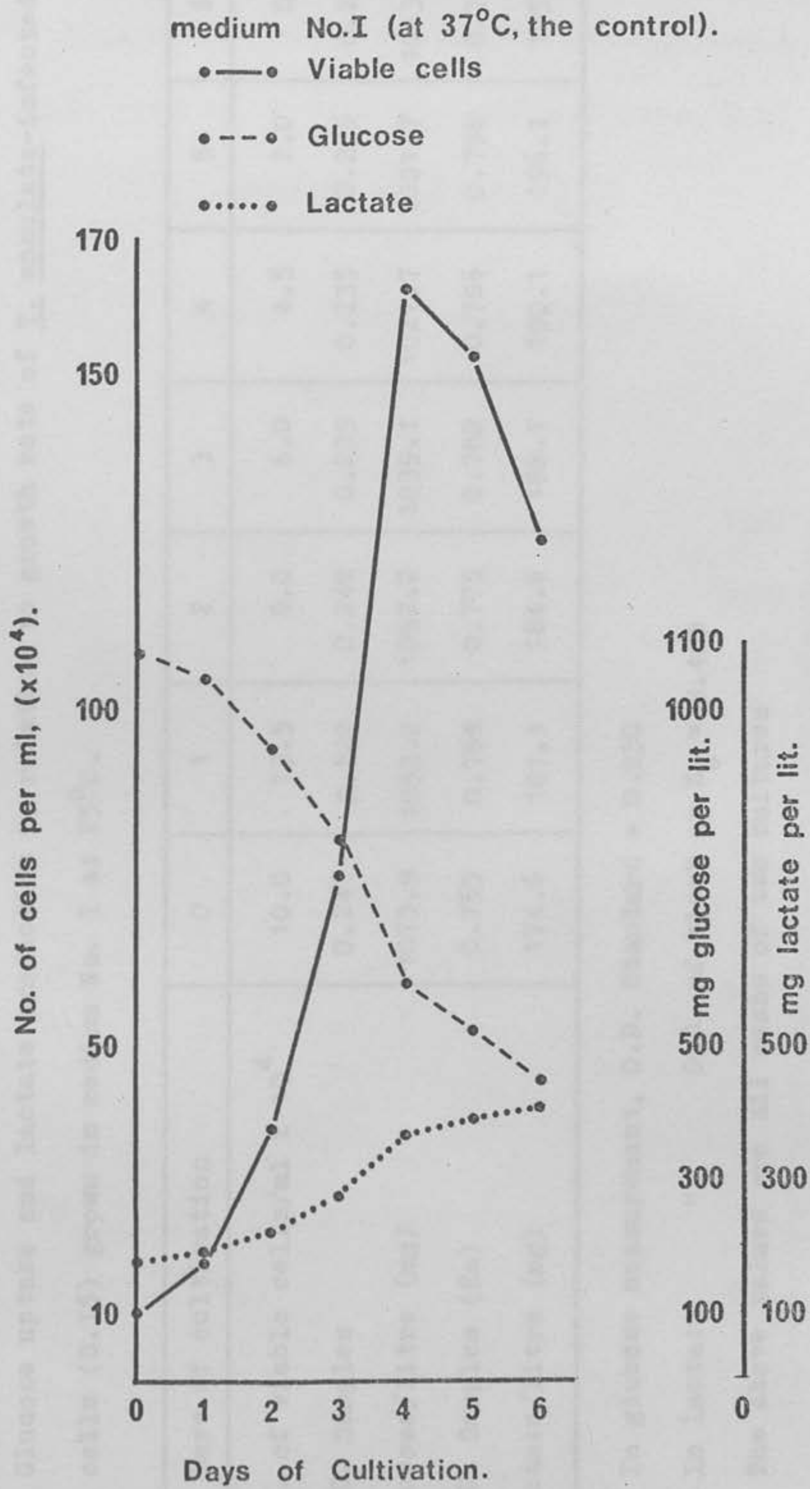


Table 8.12

Glucose uptake and lactate production in relation to growth rate of _T. annulata_-infected cells (S.15) grown in medium No. I at 25°C.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 13.5 | 9.0 | 6.0 | 4.5 | 2.0 | 0.0 |
| O.D. Samples | 0.247 | 0.242 | 0.242 | 0.239 | 0.235 | 0.235 | 0.233 |
| Glucose/litre (mg) | 1073.9 | 1052.2 | 1052.2 | 1039.1 | 1021.7 | 1021.7 | 1013.0 |
| O.D. Samples (Es) | 0.755 | 0.768 | 0.775 | 0.782 | 0.786 | 0.798 | 0.796 |
| Lactate/litre (mg) | 174.6 | 181.1 | 184.6 | 188.1 | 190.1 | 196.1 | 195.1 |

In glucose measurement, O.D. Standard = 0.230

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures

Fig. 8·12 Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells, S.15 grown in medium No.I, at 25°C.

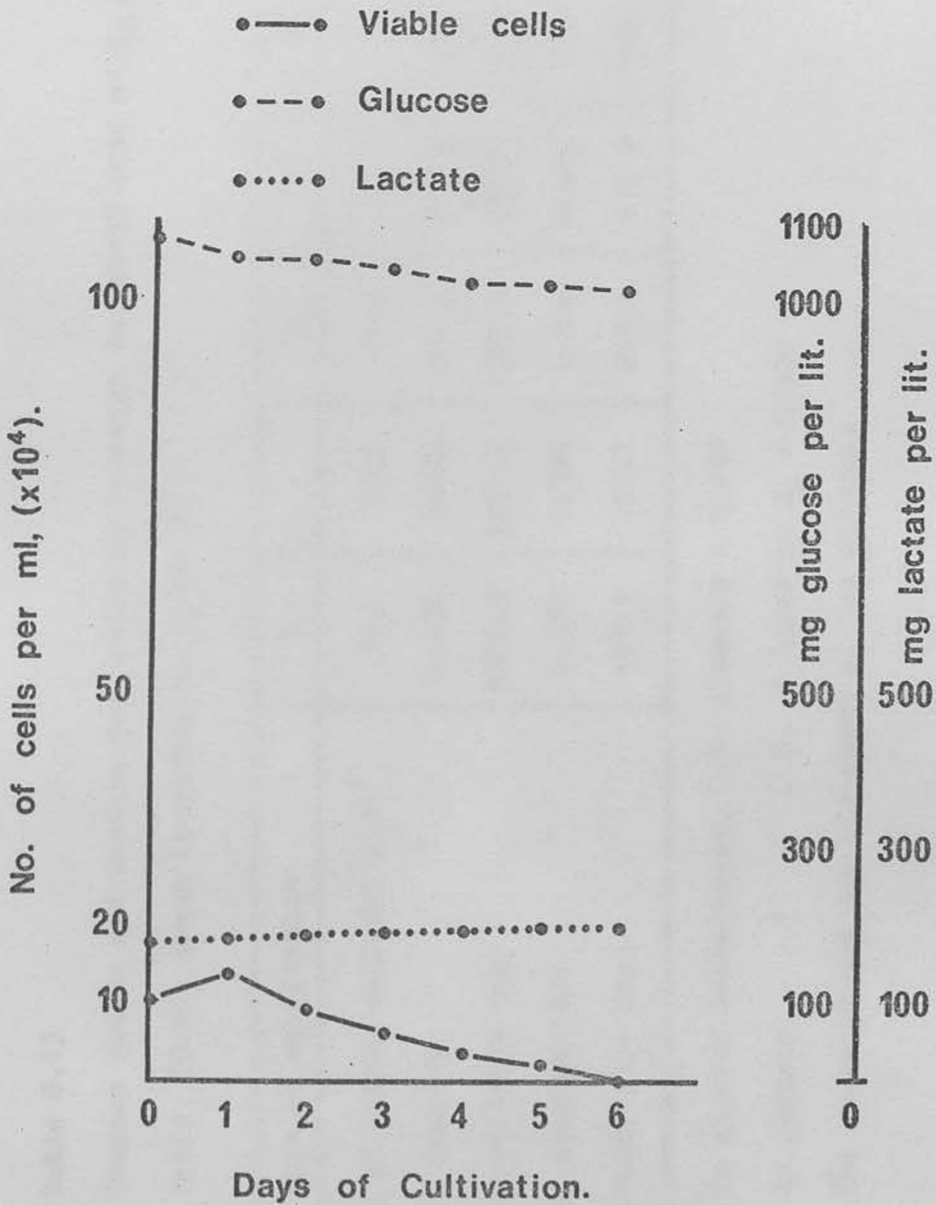


Table 8.13

Glucose uptake and lactate production in relation to growth rate of *T. annulata*-infected cells (S.15) grown in medium No. I at 35°C.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 15.5 | 20.0 | 42.5 | 55.0 | 37.5 | 12.5 |
| O.D. Samples | 0.250 | 0.242 | 0.230 | 0.216 | 0.207 | 0.200 | 0.198 |
| Glucose/litre (mg) | 1086.9 | 1052.2 | 1000.0 | 939.1 | 900.0 | 869.6 | 860.9 |
| O.D. Samples (Es) | 0.760 | 0.786 | 0.836 | 0.877 | 0.905 | 0.917 | 0.920 |
| Lactate/litre (mg) | 177.1 | 190.1 | 215.1 | 235.5 | 249.5 | 255.5 | 257.0 |

In glucose measurement, O.D. Standard = 0.230

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures

Fig.8·13 Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells, S. 15 grown in medium No. I, at 35°C.

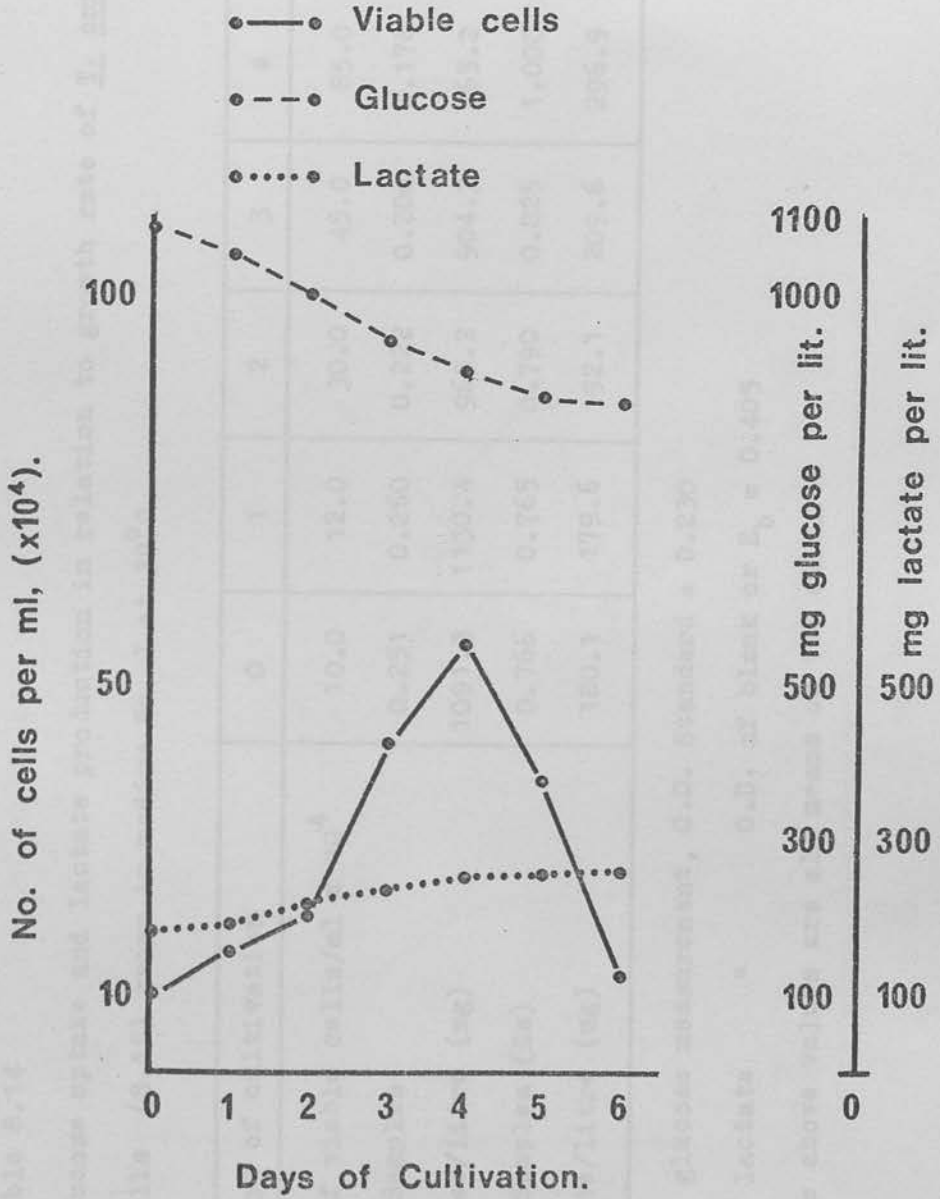


Table 8.14

Glucose uptake and lactate production in relation to growth rate of _T._ _annulata_-infected cells (S.15) grown in medium No. I at 39°C.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 12.0 | 30.0 | 45.0 | 85.0 | 97.5 | 65.0 |
| O.D. Samples | 0.251 | 0.260 | 0.222 | 0.208 | 0.176 | 0.160 | 0.156 |
| Glucose/litre (mg) | 1091.3 | 1130.4 | 965.2 | 904.3 | 765.2 | 695.6 | 678.3 |
| O.D. Samples (Es) | 0.766 | 0.765 | 0.790 | 0.825 | 1.000 | 1.033 | 1.048 |
| Lactate/litre (mg) | 180.1 | 179.6 | 192.1 | 209.6 | 296.9 | 313.4 | 320.8 |

In glucose measurement, O.D. Standard = 0.230

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures

Fig. 8·14 Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells, S.15 grown in medium No.I, at 39°C.

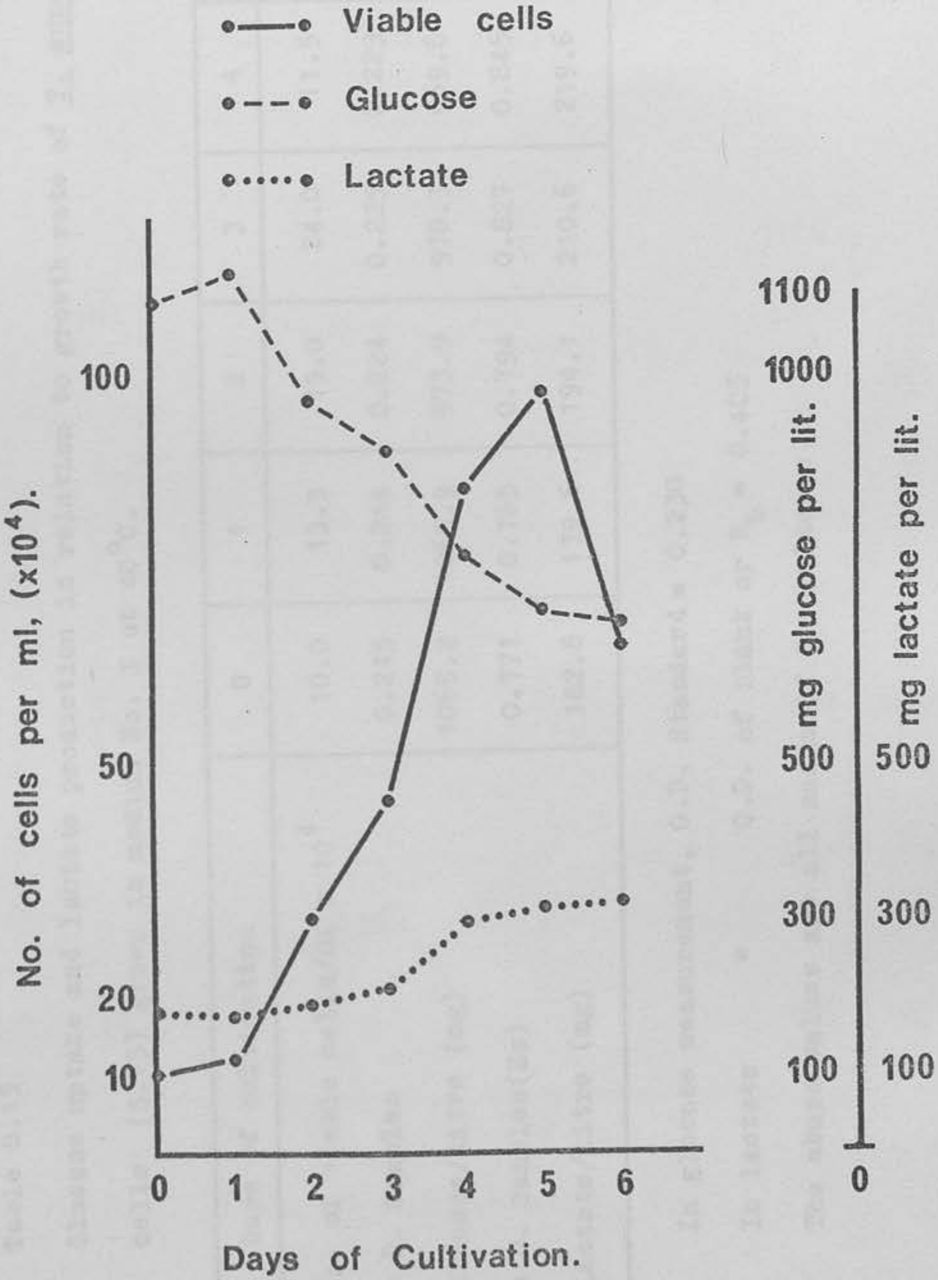


Table 8.15

Glucose uptake and lactate production in relation to growth rate of T. annulata-infected cells (S.15) grown in medium No. I at 40°C.

| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 13.5 | 19.0 | 24.0 | 11.5 | 0.5 | 0.0 |
| O.D. Samples | 0.245 | 0.244 | 0.224 | 0.225 | 0.223 | 0.220 | 0.223 |
| Glucose/litre (mg) | 1065.2 | 1060.9 | 973.9 | 978.3 | 969.6 | 956.5 | 969.6 |
| O.D. Samples(Es) | 0.771 | 0.765 | 0.794 | 0.827 | 0.845 | 0.851 | 0.860 |
| Lactate/litre (mg) | 182.6 | 179.6 | 194.1 | 210.6 | 219.6 | 222.6 | 227.0 |

In glucose measurement, O.D. Standard = 0.230

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures

Fig. 8·15 Glucose uptake and lactate production in relation to growth rate of _T. annulata_-infected cells, S.15 grown in medium No. I, at 40°C.

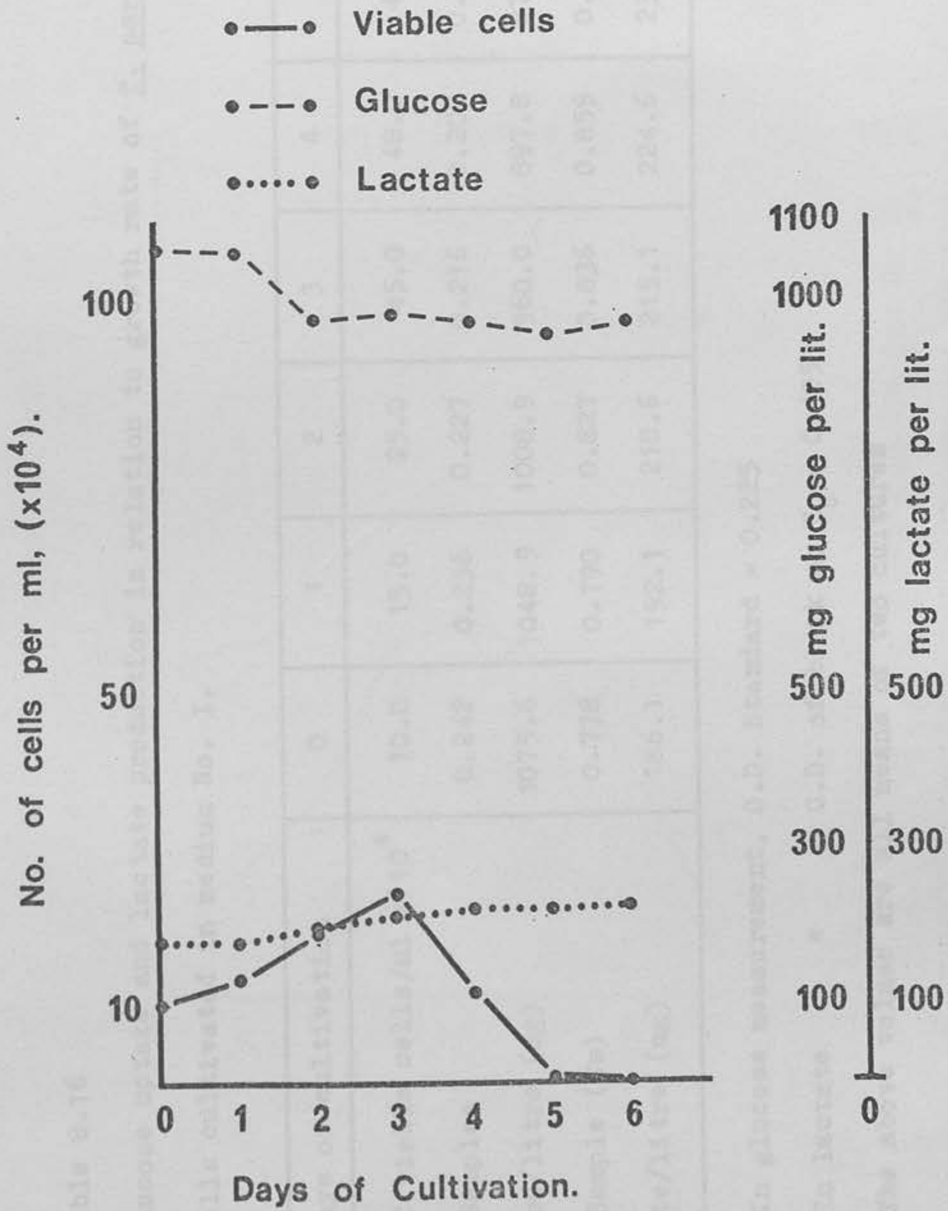


Table 8.16

Glucose uptake and lactate production in relation to growth rate of _T. parva_-infected cells cultivated in medium No. I.

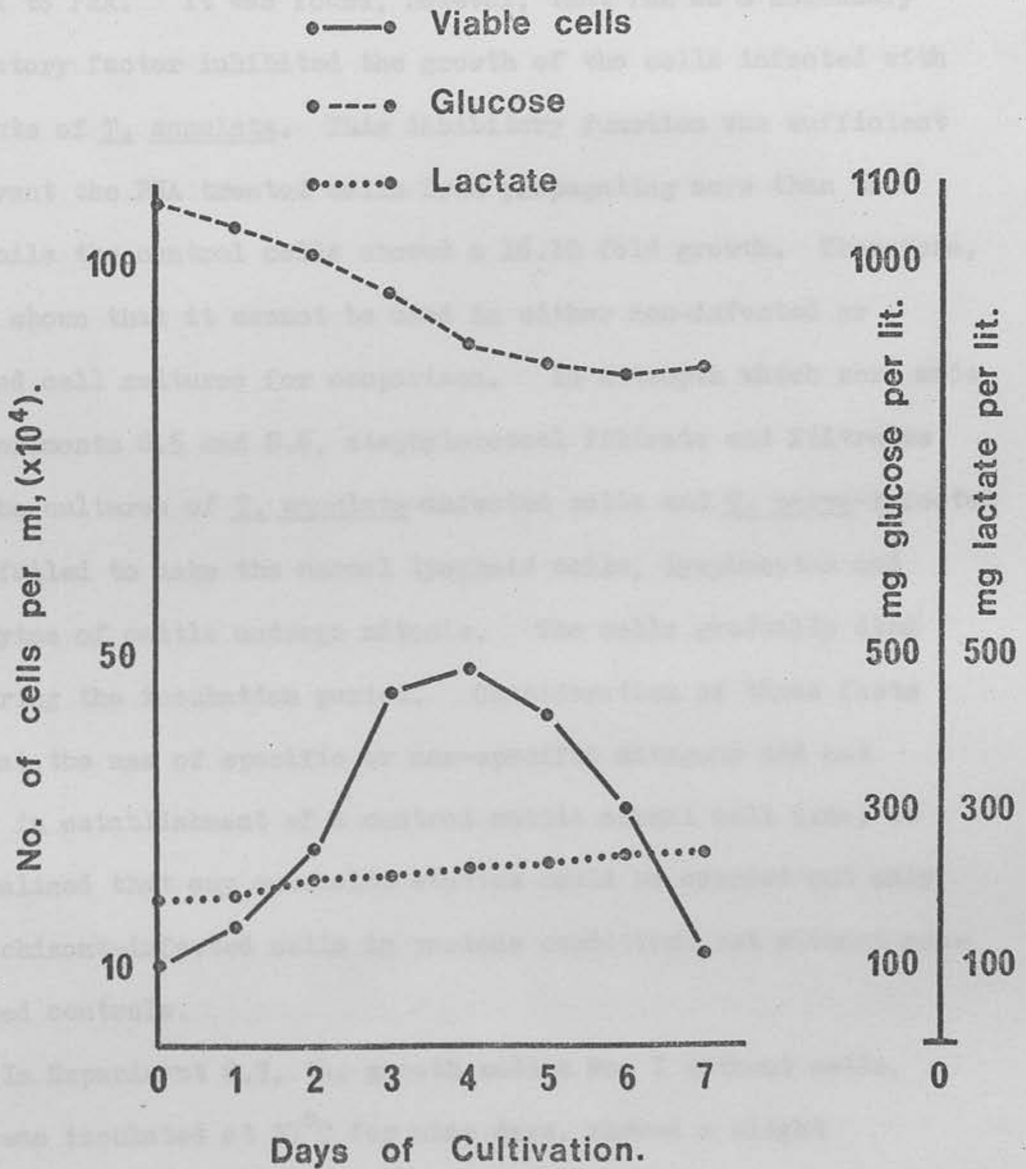
| Days of cultivation | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| No. of viable cells/ml x $10^4$ | 10.0 | 15.0 | 25.0 | 45.0 | 48.0 | 42.5 | 30.0 | 11.5 |
| O.D. Samples | 0.242 | 0.236 | 0.227 | 0.216 | 0.202 | 0.196 | 0.193 | 0.195 |
| Glucose/litre (mg) | 1075.6 | 1048.9 | 1008.9 | 960.0 | 897.8 | 871.1 | 857.8 | 866.7 |
| O.D. Sample (Es) | 0.778 | 0.790 | 0.827 | 0.836 | 0.855 | 0.868 | 0.885 | 0.898 |
| Lactate/litre (mg) | 186.1 | 192.1 | 210.6 | 215.1 | 224.6 | 231.0 | 239.5 | 246.0 |

In glucose measurement, O.D. Standard = 0.225

In lactate " O.D. of blank or $E_b$ = 0.405

The above values are all means of two cultures

Fig. 8·16 Glucose uptake and lactate production by T. parva-infected cells grown in medium No. I.



## DISCUSSION

Phytohaemagglutinin (PHA) has been widely used for stimulation of mammalian cells (Li and Osgood 1949; Hungerford, Donnelly, Nowell and Beck 1959; Moorhead et al 1960; Nowel 1960; Ling and Kay 1975). In Experiments 8.1, 8.2 and 8.3 it was shown that cattle lymphocytes from lymph node and blood, and leucocytes respond to PHA. It was found, however, that PHA as a secondary stimulatory factor inhibited the growth of the cells infected with schizonts of *T. annulata*. This inhibitory function was sufficient to prevent the PHA treated cells from propagating more than two fold while the control cells showed a 16.10 fold growth. Therefore, it was shown that it cannot be used in either non-infected or infected cell cultures for comparison. In attempts which were made in Experiments 8.5 and 8.6, staphylococcal filtrate and filtrates from the cultures of *T. annulata*-infected cells and *T. parva*-infected cells failed to make the normal lymphoid cells, lymphocytes and leucocytes of cattle undergo mitosis. The cells gradually died off during the incubation period. Consideration of these facts and that the use of specific or non-specific mitogens did not result in establishment of a control cattle normal cell line, it was realised that any metabolic studies could be carried out only with schizont-infected cells in various conditions but without non-infected controls.

In Experiment 8.7, the growth medium No. I without cells, which was incubated at 37°C for nine days, showed a slight decrease in glucose of not more than 50 mg per litre. The lactate increase in this medium was as low as 20.9 mg per litre. These results show that in the absence of cells there were negligible changes of glucose and lactate content. It is noteworthy that the

use of Hepes buffer in the medium and separate culture tubes for each sample seemed to have been effective factors for stability or minimal changes of these two components. Glucose measurement showed that the calf serum used contained 977.3 mg per litre and the LY used contained zero. Non-supplemented Eagle's MEM contained 990.9 mg glucose per litre. The reason that growth media Nos. I and II showed 1090.9 and 2081.8 mg per litre, both more than 1000 and 2000 mg per litre, is that they had been made up with ten per cent calf serum v/v. The schizont-infected cells used in Experiment 8.8 trial (a) which showed a mean of 19.75 fold growth utilised 801.9 mg out of 1094.3 mg glucose per litre. This suggests a highly active glucose consumption by these cells as 73.27 per cent of the original glucose amount in the medium was used. The lactate produced was 284.4 mg per litre. There is a close correlation between cell growth and glucose uptake and between cell growth and lactate production. In trial (b) of the previous experiment, i.e. with the medium II, the schizont-infected cells increased 19.25 fold in number, used up 754.8 mg glucose out of 2160.4 mg and produced 240.5 mg lactate. It seems that a surplus of glucose in growth medium does not affect the glucose utilisation by the cells. As in the previous trial (a) there are close correlations between cell growth and glucose consumption and between cell growth and lactate production. In Experiment 8.9, trials (a) and (b) when schizont-infected cells of S.15 were compared with the cells of S.3, the differences are as follows:- (a) The cells of S.15 reached a mean increase of 18.85 fold, utilised 724.5 mg glucose and produced 269.5 mg lactate. The cells infected with S.3 in trial (b) showed a 9.5 fold growth, utilised 408.8 mg glucose and produced 125.3 mg

lactate per litre. The difference between the growth rates in trials (a) and (b) and consequent differences between glucose uptake and lactate production can be explained as due to the different growth potentials of the two strains which were demonstrated in Chapter 5. In both trials there were correlations between glucose consumption and lactate production in relation to the cell growth. Consumption of glucose and production of lactate, at various temperatures (Experiment 8.10), differed considerably. At 37°C, in trial (a), glucose uptake and lactate production were at the highest levels, 634.8 mg and 230.6 mg per litre, respectively. This can be explained as being due to the high growth rate, 16.25 fold, at this temperature. Glucose uptake and lactate production at 35°C in trial (c) were 226.0 mg and 79.9 respectively, corresponding to a lower growth rate, 5.50 fold. The same equation appeared in trial (d) when the schizont-infected cells showed 9.75 fold increase and resulted in 413.0 mg glucose uptake and 140.7 mg lactate production. In both trials (b) and (e) when the cells were cultivated at 25°C and at 40°C, the growth rates appeared not more than 1.35 fold and 2.40 fold, respectively. These poor growths of the cells, as might be expected, correlate with the low levels of glucose uptake and lactate production; so that in trial (b) glucose uptake was only 60.9 mg and lactate production was only 20.5 mg per litre. The two above values in trial (e) were not more than 108.7 mg and 44.4 mg per litre, respectively. It was understood that ranges of glucose uptake and lactate production correlate with rate of cell growth which is governed by differences in strain (Experiment 8.9) or species of the parasite (Experiment 8.11). In the latter experiment schizont-infected cells of _T. parva_ presented a 4.8 fold growth

and resulted in 217.8 mg glucose uptake and 59.9 mg lactate production.

# CHAPTER 9

## CRYOPRESERVATION AND RETRIEVAL OF SCHIZONT-INFECTED CELLS

Cryopreservation is undoubtedly one of the most practical means by which many organisms including *Theileria* species can be maintained for use in *in vitro* and *in vivo* studies. Some of the factors involved in the preservation and retrieval of schizont-infected cells at low temperatures have been investigated.

Experiment 9.1

Selection of the type and concentration of cryoprotectant for optimum freezing and thawing of culture samples.

Two cryoprotectants, glycerol and dimethyl sulphoxide (DMSO) have been used successfully for preservation of lymphoid cells infected with *Theileria* spp., by a number of authors (see Chapter 2). In several preliminary trials different concentrations of these cryoprotectants were examined and finally two concentrations, 7.5 and 10.0 per cent v/v, appeared to be superior. Therefore, these two concentrations of each cryoprotectant were compared. The medium used for cell preservation was Eagle's MEM with Hank's salts supplemented with CS and LY, as described in Chapter 3. The pH of the medium was adjusted to 7.2, using Hepes buffer and sodium bicarbonate. Schizont-infected cells were harvested from the culture in the third or fourth day of cultivation. These were centrifuged at 400 G for seven minutes and the deposited cells were resuspended in fresh medium. The number of cells was adjusted to 4.0 x $10^6$ per ml, i.e. twice the desired cell

concentration. Glycerol and DMSO were mixed with fresh medium at twice the final concentrations as follows:- (a) and (b) 15.0 per cent and 20.0 per cent glycerol, respectively; and (c) and (d) 15.0 per cent and 20.0 per cent DMSO, respectively. Five ml aliquots of these were, then, added to equal volumes of the cell suspension, separately dropwise, over a period of five minutes while the containers were gently shaken. This was carried out according to Meryman (1968) and Rowe (1970) in order to minimise any possible osmotic shock to the cells. The numbers of cells were, thus, adjusted to $2.0 \times 10^6$ per ml of medium with 7.5 and 10.0 per cent of each cryoprotectant, respectively. Five more minutes were allowed for equilibration of the cryoprotectants to occur inside and outside the cells at room temperature. The cell suspensions were dispensed in Bijou bottles in two ml aliquots and these were capped tightly. The bottles were wrapped in cotton-wool and placed in a solid carbon dioxide cabinet at -78°C for slow uncontrolled cooling (Dalgliesh 1972; Cunningham _et al_ 1973 and Hooshmand-Rad 1973). After seven days the stabilates were taken out for examination. The bottles were shaken in a water bath at 37°C and the stabilates were thawed by the rapid method. Samples were taken immediately after thawing and counts were made for viable and non-viable cells. Eight ml of fresh medium were added dropwise over a period of five minutes to each two ml aliquot of the thawed stabilates of all samples. The samples were then centrifuged at 160 G for ten minutes. The supernates were discarded and the cell deposit of each group was resuspended in fresh medium made up to ten ml. Viable cells were counted at this stage, also. These were adjusted to $200.0 \times 10^3$ cells per ml of medium and were set up at 37°C for re-establishment of cultures.

RESULTS

The results of all four trials (a), (b), (c) and (d) were generally satisfactory. The percentages of viable cells recovered after elution of the cryoprotectants from the cells were: 66.25 for (a), 60.75 for (b), 61.00 for (c) and 65.00 for (d), as shown in Table 9.1. Schizont-infected cells recovered in all four trials were established.

While there was no marked difference between the results with the two cryoprotectants at two levels each, in the following experiments 7.5 per cent glycerol was used as cryoprotectant as the above tests had shown it to be satisfactory and convenient.

Experiment 9.2

Re-establishment of frozen and thawed stabilates in relation to numbers of seed-cells per ml for initial cultivation.

In routine work, when thawed stabilates were used for re-establishment with different seeding rates, variations in the success of re-establishment were observed. The reason for this was investigated in this experiment. Schizont-infected cells were preserved at low temperature, exactly as in the previous experiment using 7.5 per cent glycerol as the cryoprotectant. The recovered cells from thawed stabilates were treated and cultivated as before but with the following seeding rates: (a) $50.0 \times 10^3$, (b) $100.0 \times 10^3$, (c) $200.0 \times 10^3$ and (d) $400.0 \times 10^3$ cells per ml of medium. The medium and all conditions of cultivation were the same as in the previous experiment. The cells were counted daily for four days, after which, sub-culture was carried out.

Table 9.1

Recovery rates from frozen and thawed stabilates of schizont-infected cells with different concentrations of glycerol and DMSO.

| Trials | Cryoprotectants % v/v | No. of cell/ml x $10^3$ | | % Recovery of viable cells | No. of cells/ml x $10^3$ after elution | % Recovery of viable cells |
|---|---|---|---|---|---|---|
| | | before freezing | after thawing | | | |
| a | 7.5 Glycerol | 2000 | 1400 | 70.00 | 1325 | 66.25 |
| b | 10.0 " | 2000 | 1350 | 67.50 | 1215 | 60.75 |
| c | 7.5 DMSO | 2000 | 1310 | 65.50 | 1220 | 61.00 |
| d | 10.0 " | 2000 | 1420 | 71.00 | 1300 | 65.00 |

Above values represent means of cell counts from duplicate samples.

RESULTS

The results are shown in Table 9.2.

(a) The cells diminished in three days and the culture did not establish.

(b) The cells showed a decline after 24 hours but afterwards grew slowly and established themselves.

(c) The cells grew fairly steadily and established themselves.

(d) The growth was faster and re-establishment was quicker.

Table 9.2

Re-establishment of frozen and thawed stabilates in relation to numbers of seed-cells per ml of medium.

| Trial | Days of cultivation | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| a | 50 | 25 | 15 | 5 | 0 |
| b | 100 | 65 | 80 | 140 | 320 |
| c | 200 | 250 | 360 | 780 | 950 |
| d | 400 | 480 | 700 | 940 | 1050 |

No. of cells per ml, x $10^3$

Above values represent means of cell counts from duplicate cultures.

Experiment 9.3

The use of different containers in low temperature preservation of schizont-infected cells.

Schizont-infected cells with 7.5 per cent glycerol as cryo-protectant were dispensed in two ml aliquots in (a) two ml ampoules,

(b) Bijou bottles with siliconised cap liners, (c) Bijou bottles with the type of metal caps which are perforated for needle insertion and lined with red or black rubber, (d) four ml polythene bottles with hard plastic screw caps without liners. The pH of the cell suspension was adjusted to 7.2 as before. The ampoules were sealed over a gas flame. The other bottles were stoppered tightly. Duplicate samples were stored at -78°C as described in Experiment 9.1. The stabilates were thawed after three weeks and examined as follows:- (i) The pH of each group was measured. (ii) Counts of viable cells were carried out after thawing and elution of glycerol. (iii) Cell cultures were carried out from each group, as described in Experiment 9.1.

RESULTS

The results are shown in Table 9.3. In (a) and (b) changes of pH were slight, the numbers of recovered cells were high and both established in culture satisfactorily. (c) The pH decreased from 7.2 to 6.7, cell recovery was low (34.0 per cent of the initial number) and re-establishment was achieved but with some difficulty. (d) The pH fell from 7.2 to 5.6, the cell recovery was very low (less than ten per cent of the initial cell number) and the recovered cells did not establish. It was noted that when the tubes of group (d) were removed from the low temperature cabinet, the caps were slightly loose.

Experiment 9.4

Storage of schizont-infected cells at -24°C.

Schizont-infected cells were prepared in several samples in growth medium containing 7.5 per cent v/v glycerol and 2.0 x $10^6$

Table 9.3

Recovery rates of schizont-infected cells using different containers for cryopreservation.

| Trials | pH after thawing | No. of cells/ml x $10^3$ | | % recovery of viable cells | No. of cells/ ml x $10^3$ after elution of glycerol | % recovery of viable cells |
|---|---|---|---|---|---|---|
| | | Before freezing | After thawing | | | |
| (a) | 7.0 | 2000 | 1420 | 71.0 | 1340 | 67.0 |
| (b) | 7.0 | 2000 | 1410 | 70.5 | 1360 | 68.0 |
| (c) | 6.7 | 2000 | 820 | 41.0 | 680 | 34.0 |
| (d) | 5.6 | 2000 | 260 | 13.0 | 180 | 9.0 |

pH before freezing = 7.2

(a) Ampoule

(b) Bijou bottle with silicone liner

(c) " " " perforated cap and rubber liner

(d) Polythene bottle with hard plastic cap without liner

Above values represent means of cell counts from duplicate samples.

cells per ml, as described before. The samples used were in Bijou bottles and sealed ampoules. These were placed in the deep freeze compartment of a refrigerator at -24°C. At six, 24, 30 and 48 hours, duplicate samples of each were taken, thawed and examined as before. Cell counts and cultivation tests were carried out. Corresponding controls were kept at -78°C.

RESULTS

The control stabilates showed the same picture as in the previous experiments. The cells of experimental samples showed a marked decrease. The mean recovery rates of viable cells from two Bijou bottles were: 42.5, 18.0, 5.0 and zero per cent, after six, 24, 30 and 48 hours, respectively. Average numbers of recovered viable cells from the ampoules were similar to those from Bijou bottles. Only the samples thawed after six hours were successful in re-establishment. This experiment was repeated several times even with various concentrations of both glycerol and DMSO but the results were equally unsatisfactory.

Experiment 9.5

Recovery of schizont-infected cells frozen and kept at -78°C or -150°C and re-stored at -24°C.

Samples of schizont-infected cells containing $2.0 \times 10^6$ cells per ml of medium were prepared in ampoules and Bijou bottles as described before. These were kept in dry ice (approximately -78°C) and in the vapour phase of liquid nitrogen (approximately -150°C) overnight. The bottles and ampoules were then quickly transferred to storage at -24°C. Duplicate samples were taken 24 hourly and

thawed as before. The cells were counted and recovered viable cells were set up in growth medium as previously described. Cell counts and cultivation were made from control samples after low temperature treatment and before re-storage and it was shown that the preservation was satisfactory, the viable cells recovered from the deep frozen cultures before re-storage at -24°C numbering approximately 1320.0 x $10^3$/ml.

RESULTS

The results are shown in Table 9.4 and Fig. 9.1. The cells showed a rapid decline in the first 24 hours and then a slower decline in the following seven days. After eight days storage, viable cells were not more than 10.0 x $10^3$ per ml, insufficient for cultivation. Re-establishment of the cells in culture was successful with the samples re-stored at -24°C, up to seven days and was achieved readily with the samples re-stored for up to two days but, more difficulty was experienced in re-establishment with the samples re-stored for a longer period in that the growth rate was much slower. There was no difference between the samples frozen at -78°C and frozen at -150°C; and re-stored at -24°C. Replication of this experiment presented the same results. There was no marked difference between samples recovered from Bijou bottles and those from ampoules.

Experiment 9.6

Effect of storage duration on recovery rate of schizont-infected cells, preserved at -78°C and -150°C.

In routine observations on cultures of schizont-infected cells stored at low temperatures it appeared that there was little

Table 9.4

Recovery of schizont-infected cells frozen and kept at $-78^{o}C$ or at $-150^{o}C$ and re-stored at $-24^{o}C$.

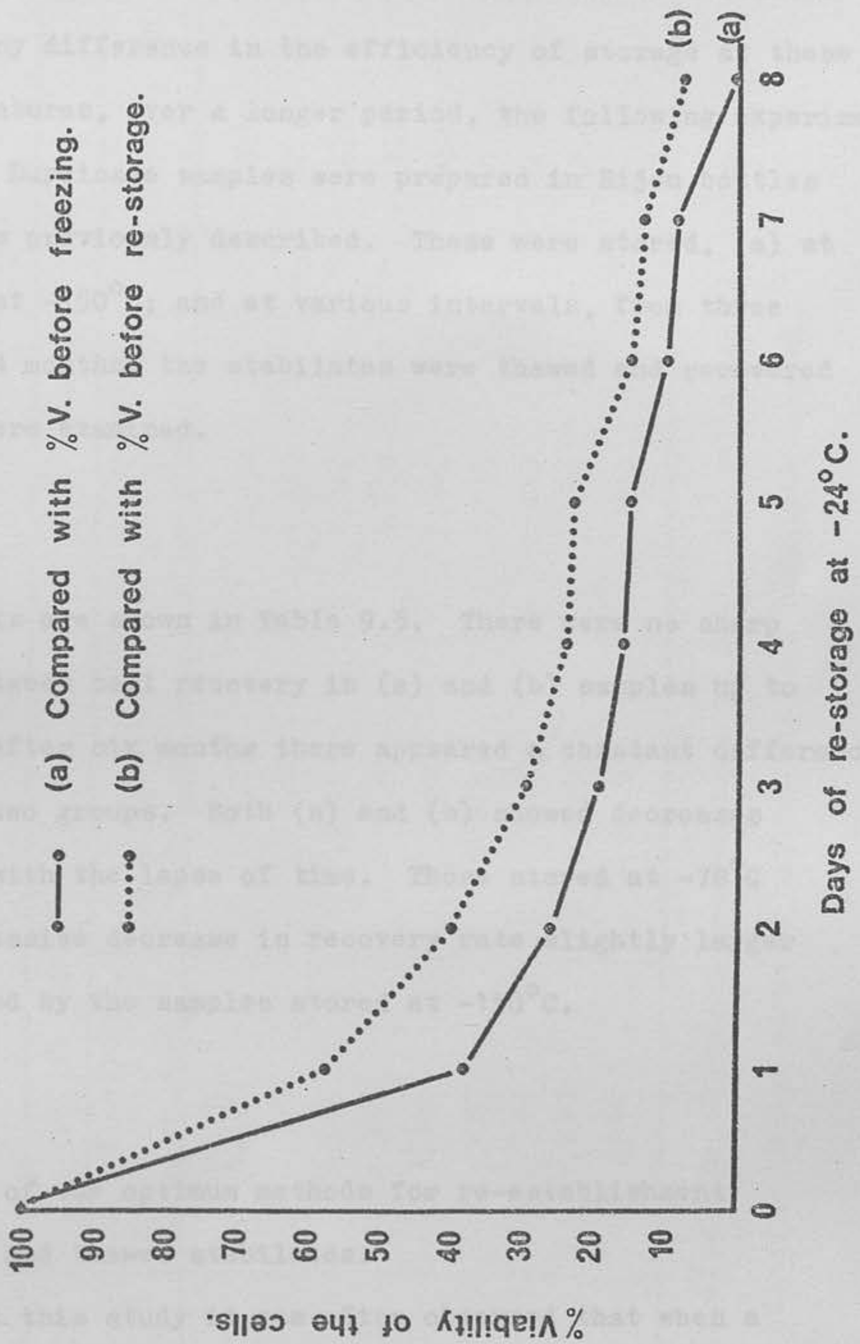
| | | Days of re-storage | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| No. of cells recovered, x $10^3$/ml | | 760 | 525 | 390 | 315 | 300 | 200 | 170 | 10 |
| Recovery of viable cells as a percentage of: | (a) original samples | 38.00 | 26.25 | 19.50 | 15.75 | 15.00 | 10.00 | 8.50 | 0.5 |
| | (b) samples before re-storing at $-24^{o}C$ | 57.58 | 39.77 | 29.54 | 23.86 | 22.73 | 15.15 | 12.88 | 0.76 |

No. of viable cells per ml before freezing = 2000.0 x $10^3$

No. of viable cells per ml before re-storage, (Day 0) = 1320.0 x $10^3$

The above values represent means of counts of duplicate samples.

Fig. 9·1 Recovery of schizont-infected cells frozen at $-78^{\circ}C$ or $-150^{\circ}C$ and re-stored at $-24^{\circ}C$.



difference in the viability of these cells on retrieval between those stored in solid carbon dioxide at -78°C and those stored in the vapour phase of liquid nitrogen at approximately -150°C, provided the period of storage was short. In order to determine if there was any difference in the efficiency of storage at these two low temperatures, over a longer period, the following experiment was designed. Duplicate samples were prepared in Bijou bottles and ampoules as previously described. These were stored, (a) at -78°C and (b) at -150°C; and at various intervals, from three months up to 24 months, the stabilates were thawed and recovered viable cells were examined.

## RESULTS

The results are shown in Table 9.5. There were no sharp differences between cell recovery in (a) and (b) samples up to four months. After six months there appeared a constant difference between these two groups. Both (a) and (b) showed decreases corresponding with the lapse of time. Those stored at -78°C showed a progressive decrease in recovery rate slightly larger than that showed by the samples stored at -150°C.

## Experiment 9.7

Selection of the optimum methods for re-establishment of frozen and thawed stabilates.

Throughout this study it was often observed that when a frozen samples was thawed, the higher the percentage viability of the cells, the more successful the re-establishment of schizont-infected cells and *vice-versa*, irrespective of the total numbers of cells. Variation in the success of re-establishment of thawed

Table 9.5

Effect of storage duration on recovery rate of schizont-infected cells cryopreserved at -78°C and -150°C.

| Storage duration (months) | (a) at -78°C | | (b) at -150°C | |
|---|---|---|---|---|
| | No. of cells/ml, x $10^3$ after elution | % recovery of viable cells | No. of cells/ml, x $10^3$ after elution | % recovery of viable cells |
| 3 | 1,400 | 70.00 | 1,430 | 71.50 |
| 4 | 1,360 | 68.00 | 1,350 | 67.50 |
| 6 | 1,265 | 63.25 | 1,320 | 66.00 |
| 8 | 1,170 | 58.50 | 1,250 | 62.50 |
| 12 | 1,040 | 52.00 | 1,200 | 60.00 |
| 15 | 970 | 48.50 | 1,015 | 50.75 |
| 18 | 720 | 36.00 | 890 | 44.50 |
| 22 | 650 | 32.50 | 880 | 44.00 |
| 24 | 610 | 30.50 | 830 | 41.50 |

Viable cells before freezing were adjusted to 2000 x $10^3$ cells per ml medium.

Above values represent means of cell counts from duplicate samples.

samples was observed when per cent viability was very low, i.e. 15 per cent or lower. To determine whether or not this could be due to the presence of non-viable cells this experiment was designed. A frozen sample of schizont-infected cells (S.15) was thawed and cell counts were carried out. The per cent viability of the sample was 12.5, after elution of glycerol. Cultures were set up from these cells in three ways:-

(a) Duplicate cultures in 25 $cm^2$ T-flasks using $200.0 \times 10^3$ schizont-infected cells per ml of growth medium.

(b) Duplicate cultures with the same number of cells per ml in the same type of culture vessel, but sloped at an angle of approximately 15 degrees, (deep culture).

(c) Viable cells were separated from the non-viable cells, using Lymphoprep as described in Chapter 3. The viability of the final cell suspension was increased from 12.5 to 68.0 per cent. From this cell suspension duplicate cultures were set up with the same number of cells per ml, in the same type of culture vessels as in (a) and (b) above. The growth medium and other conditions of cultivation were the same as described in Experiment 9.1. These cultures were passaged every three days.

RESULTS

(a) None of the cultures established.

(b) One of the cultures established and after 15 days reached a per cent viability of 62.5.

(c) Both of the cultures established. The one incubated in the sloped position,after 9 days and the second, in the horizontal position, after 12 days exceeded a viability of 70.0 per cent.

Separation of viable cells from non-viable by means of

Lymphoprep was repeated several times and it was found possible to purify a cell suspension of non-viable cells up to 95.5 per cent viability. Replication of this experiment showed the same results, Figs. 3.3 and 3.4.

DISCUSSION

Low temperature storage of pathogens is of great importance in research and is now accepted as an essential process in biological laboratories. Considerable advances have been made in the knowledge and use of cryopreservation as applied to protozoal pathogens which proved more refractory to low temperature storage methods than viruses and bacteria owing to the effects of freezing on the comparatively large bodies of cytoplasm involved. Thus, the methods used in cryopreservation of protozoal parasites have been the subject of much investigation by many authors, for example, Meryman (1968), Mazur (1970) and Pegg (1976). In these methods a balance has to be established between damage due to freezing and low temperature preservation of cells which can be retrieved in the viable state. Successful methods for the preservation of schizont-infected cells have assumed even greater importance since Theileria spp. have now been adapted to tissue culture and transformed lymphoblasts have come into use as vaccines.

Success in the freezing of Theileria schizont-infected cells has been closely linked with the use of substances which act as cryoprotectants and with rates of cooling and methods of thawing. Cryopreservation of T. annulata schizonts has been described by Tsur and Pipano (1962), Hulliger (1965), Hashemi-Fesharki and Shad-Del (1973a and 1973b) and Hooshmand-Rad (1973) but further knowledge on the process involved appeared necessary and the

experiments in this chapter were designed to define the values of various parameters in this context.

In Experiment 9.1 the results showed that both glycerol and DMSO are satisfactory cryoprotectants. Taking into account such considerations as convenience and simplicity in use and the apparent marginally better effect of glycerol and its lack of toxicity (Stecher, Windholz, Leahy, Bolton and Eaton 1968 and Pegg 1976), the latter was used at a level of 7.5 per cent in the subsequent work. The numbers of viable infected cells used in the initiation of a culture have been recognised for some time as affecting the establishment and growth rate of cultures. In Experiment 9.2, cultures were set up with four levels of seeding from $50.0 \times 10^3$ to $400.0 \times 10^3$ infected viable cells per ml. It was shown that more than $50.0 \times 10^3$ cells per ml are necessary for successful establishment but that while $100.0 \times 10^3$ cells per ml were sufficient for establishment, higher seeding rates gave more rapid growth rates. Seeding rates of $200.0 \times 10^3$ and $400.0 \times 10^3$ cells per ml gave very satisfactory results.

Experiment 9.3 was designed to indicate whether or not the type of container could have an effect on the efficiency of low temperature storage of theilerial cultures. The best results were obtained with sealed ampoules and Bijou bottles with siliconised liners. The containers with perforated caps and rubber liners gave an unexpectedly poor result. As these containers were stored in dry ice, the possibility of penetration of carbon dioxide through the rubber liners can not be discarded. Busby, House and Macdonald (1964) have stated that this penetration can take place. The polythene bottles with hard plastic caps gave very poor results but this could be attributed to the loosening

of the caps in the dry ice. This was probably due to the difference in the co-efficient of expansion of the polythene of the bottle and the hard plastic of the cap. Penetration of the bottle by carbon dioxide was thus facilitated.

The temperatures at which cultures of T. annulata-infected cells are stored are important relative to the transport and use of vaccines in the field. Experiment 9.4 demonstrated quite clearly that a temperature of -24°C was quite unsuitable for practical storage.

In Experiment 9.5 the possibility of transportation of schizont-infected cells, which were already preserved in low temperature then re-stored at -24°C prior to shipment, was investigated. In contrast with the results of Experiment 9.4, it was surprisingly observed that viable infected cells survived for up to seven days in a re-storage temperature of -24°C, the numbers of viable cells being decreased within this period. The viable cells which survived after seven days were still sufficient in number for successful re-establishment. Corresponding infected cells re-stored for up to six days could provoke mild reactions of theileriosis in susceptible calves, which resisted challenge tests (unpublished data 1973b).

In Experiment 9.6 the effect of the duration of storage on the recovery rate of viable infected cells was shown over a period of four to 24 months. Cryopreservation was at two levels, -78°C in dry ice and -150°C in the vapour phase of liquid nitrogen. After three months of storage there was little difference between recovery of cells from samples at -78°C and -150°C. Thereafter there was a decline in viability which was more rapid in those cells from -78°C storage than in those stored at -150°C. This

was marginal over the period of 24 months indicating that for this period of storage a sufficient proportion of viable cells for culture re-establishment was present, irrespective of which of the two temperatures were used.

Re-establishment of the preserved schizont-infected cells which retained low percentage viability was investigated in Experiment 9.7. Establishing such a cell culture in deep culture was brought about by encouraging the viable cells to clump and to adhere, both of which favoured growth. Another factor having a favourable influence on the establishment of cultures was the separation of viable from non-viable cells by means of Lymphoprep and the reconstituting of suspensions of higher percentage viability.

# CHAPTER 10

## GENERAL DISCUSSION AND CONCLUSIONS

In the introduction to this thesis it was shown that successful cultivation of _Theileria annulata_ as the macroschizont in lymphoblast cells has been carried out for a considerable period. Early work in the field showed that such cultures could bring about the attenuation of the virulence of the parasite and that these cultures could be re-established in susceptible cattle causing mild episodes of disease followed by a greater or lesser degree of immunity. This potential was very quickly exploited as a means of artificial immunisation and various strains of _T. annulata_, adapted to tissue culture, were used as experimental vaccines. The ready facility with which such strains could be adapted to tissue culture, their attenuation and their successful use as immunogens were probably responsible for the fact that many of the aspects of their tissue-culture characteristics were not fully studied. The work described in this thesis covers some of these neglected aspects and has shown the response of the parasite to various changes brought about in the tissue culture environment.

In order to facilitate this work it was necessary to standardise the techniques used and to select strains which could be expected to lend themselves most readily to the studies envisaged. These factors, basic to the major experimental work, are described in Chapter 3, "Materials and methods". From this chapter the most important features which emerged, concerned the strains of parasite, the media used and the methods of examining the infected cells microscopically. Of the strains of _T. annulata_ available, the most satisfactory for the experimental

work were S.3 a virulent strain, approximating to a wild strain except that it does not produce piroplasmic forms, and S.15 a milder apiroplasmic strain very well adapted to cultivation *in vitro*. Experiments with various media and supplements led to the selection of those based on Eagle's MEM with either Hank's or Earle's salts. Both of these were supplemented with calf serum (CS) and lactalbumin hydrolysate and yeast extract (LY).

In smears from cell cultures it was found that irregular and unsatisfactory preparations were obtained by the normal biopsy smearing methods. Good results were obtained by expelling the suspension of infected cells onto slides in a linear manner from a capillary tube. An even better picture was obtained when washed bovine red cells were added to the suspension. However, to avoid any complications which might arise from the addition of extraneous cells to cultures, the linear smear made from capillary tubes was used for all microscopic preparations except for those prepared essentially for karyotypic analysis.

Chapter 4 of this thesis is devoted to the methods of isolation of the strains used in this work. S.3 was adapted to tissue culture by the author who also adapted three other strains, S.19, S.20 and S.21, the cultivation of which is compared with that of S.15 and S.3 in Chapter 5. S.15 which had previously been isolated and adapted to tissue culture was re-isolated from the blood of calves infected from preserved stabilates by the author who demonstrated, for the first time, a satisfactory method of isolating strains of *T. annulata* *in vitro* from whole blood.

The growth of schizont-infected lymphoblasts *in vitro* was studied in an endeavour to determine the optimum conditions for

propagation of the parasites. Many variations of media and supplements were used and growth rates in a wide range of conditions were investigated. In these experiments the five strains of T. annulata (S.3, S.15, S.19, S.20 and S.21) were used. There was a difference in facility to grow which appeared to correlate with the virulence of the strains as demonstrated *in vivo*. The more virulent the strain, the less well did it grow. This was in accord with the findings of Hooshmand-Rad and Hashemi-Fesharki (1968) who used three strains, S.11, S.15 and S.3.

In this series of experiments it was confirmed that for optimum growth, the most satisfactory medium was Eagle's MEM with Hank's or Earle's salts and CS and LY. The optimum pH for growth was 7.0, the best buffering system being sodium bicarbonate in association with Hepes. The rate of growth was shown to be linked with the numbers of seed-cells, greater numbers being required for establishment of S.3 than for S.15. A minimum seeding rate of $10.0 \times 10^4$ cells per ml for S.3 and $5.0 \times 10^4$ cells per ml for S.15 was demonstrated. Furthermore, the facility of establishment of new cultures was shown to be dependent on the proportion of cells in the seeding suspension which were viable. The higher the percentage of viable cells, the more rapidly did the cultures establish and propagate. Some other environmental factors in connection with growth rate were also investigated. The rate of growth was higher in stationary culture compared with that in stirred culture, presumably as it facilitated settlement and growth of the cells. The type of culture vessels appeared to have an effect on the rate of growth, as plastic vessels yielded a greater number of cells than did glass vessels. This could

have been due to the fact that the cells adhered more readily to the plastic vessels. A wide range of incubatory temperatures which was tested showed that 37°C was the optimum for growing schizont-infected lymphoblasts. It was noted that the presence of a feeder layer facilitated establishment of cultures in those strains in which difficulty was encountered in such establishment. The feeder layer could be formed of completely extraneous cells such as BHK cells or of bovine fibroblasts, either preformed or developing at the time of isolation.

In connection with morphology of the macroschizont-infected lymphoblasts, experimental work revealed that in regularly growing cultures of _T. annulata_, one theilerial body per cell is the usual number. In such active cultures the number of schizont particles appeared commonly to be five to 20 per cell with an overall mean number of 13.01.

Throughout the examination of stained smears of cultures it was often observed that the theilerial body divided by binary fission synchronously with cell mitosis, as reported by Hulliger _et al_ (1964).

Attempts made to bring about the development from the macroschizont to the later stages in the cycle, e.g. the microschizont, micromerozoite and the intra-erythrocytic parasite, are described in Chapter 6. These attempts employed variations in culture conditions which might be related to the changes which occur _in vivo_ and which are associated with similar changes in the parasite.

The factors resulting in the appearance of definite microschizonts were high incubatory temperature (40°C to 42°C), the addition of normal bovine lymph cells, circulating WBC and rbc, the addition of lymph node extract (LNE) and calf lymph. These

factors caused developmental changes of the macroschizonts to the microschizonts in both strains, S.15 and S.3. In addition, some other factors such as $CO_2$/oxygen tension in culture and decrease of pH of the medium appeared to bring about small numbers of microschizonts in S.3 only. Considering the fact that variation of $CO_2$/oxygen tension and of pH in the medium caused developmental changes only in the cultures of S.3 and that application of the former factors caused more frequent microschizonts in S.3 than in S.15, it appears that S.3 undergoes the changes from the macroschizont form more readily than does S.15. This might be due to the greater facility for adaptation and growth *in vitro* of this strain making it better able to cope with unfavourable conditions than S.3. Thus it appears to be better equipped to maintain propagation in the macroschizont stage in adverse conditions. The precursors of microschizonts, the transitional forms, appeared in association with most of the variations in conditions which were applied. However, neither of the strains of *T. annulata* (S.15 and S.3) changed beyond the microschizont stage i.e., to micromerozoites and piroplasms. Another suggestion which might be considered as contributing to the failure of development of S.15 and S.3 of *T. annulata* beyond the microschizont stages compared with *T. parva* might be the fact that these two strains do not produce piroplasmic forms *in vivo* due to the effects of a long period of artificial passage *in vivo* and *in vitro*.

The work reported in Chapter 7 was designed to explore the processes by which the theilerial schizonts propagate. Since the method of division of schizonts simultaneously with the division of the host cell was confirmed by Hulliger *et al* (1964), the

question as to whether or not multiplication can take place by any other method has remained unanswered. The early hypothesis was based on the assumption that the macroschizonts broke away from the host cell producing macromerozoites which then infected fresh lymphocytes. Attempts to bring about this process as described and discussed in Chapter 7 failed in all the experiments and no evidence was produced to support this hypothesis. While this can not be regarded as finally disposing of the hypothesis it does lend strong support to the assumption that once a schizont is established, its propagation is dependent on division synchronously with the host cell until it transforms into the microschizont.

The possibility that the whole theilerial macroschizont, having broken away from the host cell, remains viable and can be re-established in a cell of the lymphocytic series must be considered. Experiment 7.10 represented an attempt to separate whole macroschizonts (theilerial bodies) from their host cells by various means. The attempt was unsuccessful as only broken schizonts or schizonts still incorporated in their host cells could be obtained. When this material was exposed to suspensions of new cells, there was no evidence on karyotypic analysis that any transfer of parasitic material, either whole theilerial bodies or broken schizonts were taken up by new cells. Therefore, while it is impossible on this evidence to state whether or not the theilerial bodies on being released from host cells retained their viability, it appears to be unlikely. Partial support is given to this supposition by the results in Experiment 7.15 in which separated parasitic particles were introduced into completely susceptible calves without infecting the host animals.

With intracellular parasites and their host cells which are so closely interdependent as are the schizonts of the *Theileria* spp. it is impossible to determine the details of the metabolic processes of the parasites *per se* unless a suitable comparison can be made with the corresponding uninfected host cell. Even were it possible to grow uninfected lymphoblasts in culture, in parallel with the infected lymphoblasts, it would be unwise to assume that the uninfected cells would be comparable in their metabolism with the originally infected cells after the former had been carried through the same number of generations as had the infected cells. The experiments carried out in this work confirmed that continuous culture of bovine lymphoblasts *in vitro* could not be established. The necessity for glucose in culture media has been accepted generally but the levels necessary for optimum growth of *T. annulata*-infected lymphoblasts have never been determined. It was shown in Chapter 8 that an excess of glucose in the growth medium did not affect the utilisation of this nutrient by the schizont-infected cells. The glucose uptake and lactate production showed a positive correlation, the actual amounts involved being related to the rate of growth of the infected cells. This rate was shown to be governed by the strain of the parasite involved. It was similarly apparent that the uptake of glucose and the production of lactate were functions of the conditions of culture which affected the growth rate of the cells.

The work carried out on cryopreservation of *T. annulata*-infected cells showed that glycerol and dimethyl sulphoxide (DMSO) were both satisfactory cryoprotectants. For the reasons explained in the discussion of Chapter 9, glycerol was considered

to be preferable to DMSO for low temperature preservation of these cells. It was shown that for retrieval of the thawed stabilates, different strains of T. annulata require different levels of seed-cells. This is understood to be due to the potential growth of various strains, as while a seeding rate of $10.0 \times 10^4$ cells per ml was adequate for S.15, twice as many cells were necessary for re-establishment of S.3. For routine preservation of T. annulata-infected cells at low temperatures, containers such as sealed ampoules and Bijou bottles with siliconised liners which were tightly stoppered, were shown to be satisfactory as they prevent gas penetration and pH variation. A temperature of -24°C for storage of schizont-infected cells appeared to be deleterious, as possibly the freezing is not complete at this temperature. When cells were first preserved at -78°C or at -150°C and then were re-stored at -24°C (without thawing), it was found that proportions of the cells could survive up to seven days. The cells which survived for seven days on re-storage at -24°C could be retrieved in culture. The low temperature of -78°C (dry ice) was satisfactory for storage of T. annulata-infected cells provided the storage period was no more than a few months. Over longer periods, -150°C (vapour phase of liquid nitrogen) appeared to be the preferable storage temperature. When the schizont-infected cells, stored at low temperature, were thawed the lower the percentage of viable cells the more difficult was re-establishment in culture. This was presumably due to the presence of a high proportion of non-viable cells. Re-establishment of these cells, however, could be brought about in two ways, one by cultivation of the cells in sloped culture and the other by separation of viable cells

from non-viable by the use of Lymphoprep.

CONCLUSIONS

1. On preparation and staining of cell culture smears two improved methods were introduced, one linear stained smears and the other by the addition of bovine washed rbc to the cell suspension, both being satisfactory.

2. Five strains of T. annulata were experimentally isolated. Isolation of schizont-infected cells which were separated from blood, lymph nodes and liver tissues (biopsy) and from lymph nodes, liver and spleen tissues (autopsy) was readily carried out without the necessity of trypsinisation. In addition a new and simple method for isolation of the parasite from whole blood was introduced. Isolation and adaptation in tissue culture of a very virulent strain (S.3) was achieved by this method for the first time.

3. Growth rates of the five strains of T. annulata (S.3, S.15, S.19, S.20 and S.21) possessing various levels of virulence which were experimentally isolated, were compared *in vitro* and their patterns of growth established. A relationship between these patterns and the virulence of the strains was indicated. Cultivation of the five strains showed that the rate of growth *in vitro* was the highest in S.15 and the lowest in S.3.

4. The comparison of various growth media and supplements showed that Eagle's MEM with Hank's or Earle's salts both supplemented with CS and LY were most satisfactory giving the highest growth.

Lactalbumin hydrolysate and yeast extract (LY) was shown to be superior to other supplements tested.

5. The optimum pH of medium for growing _T. annulata_-infected cells was 7.0 and this was best maintained using sodium bicarbonate and Hepes.

6. Different strains of _T. annulata_-infected cells require different levels of seeding rate. These were $5.0 \times 10^4$ cells per ml for the S.15 and $10.0 \times 10^4$ cells per ml for S.3.

7. It was shown that of the temperatures tested 37°C was the optimum for the cultivation of strains of _T. annulata_-infected cells _in vitro_.

8. The schizont-infected cells grew more efficiently in stationary culture than in stirred culture. These cells also grew better in plastic culture vessels than in glass vessels.

9. There was a positive correlation between the percentage of viable cells in schizont-infected suspension and the rate of growth. In regularly passaged cultures, the highest percentage of viable cells appears on the third or fourth day of cultivation.

10. In establishment of cultures of refractory strains, the association of a feeder layer of cells such as BHK cells is helpful.

11. In active and regularly passaged cultures, the majority of

macroschizont-infected cells contain one theilerial body and a small minority show two to five bodies.

12. The numbers of schizont particles in the majority of host cells were from five to 20 with an overall average of 13.01 per cell.

13. Forms of the parasite beyond the macroschizont stage were developed in culture by certain variations in cultural environment. In no case, however, could these changes be brought about to a marked degree.

14. No evidence was produced for the hypothesis that propagation of Theileria annulata schizonts can take place by the transfer of schizonts or schizont particles to uninfected cells. The schizont particles separated from tissue culture also failed to infect susceptible calves.

15. Normal bovine lymphoid cells, blood leucocytes and spleen cells failed to establish themselves in tissue culture as continuous cell lines with or without specific or non-specific mitogens. There was a positive correlation between glucose uptake and lactate production in culture and the growth rate of schizont-infected cells. A surplus of glucose in growth medium did not affect the utilisation of this nutrient by the schizont-infected cells.

16.

(a) For preservation of schizont-infected culture cells, glycerol

and dimethyl sulphoxide (DMSO) were both satisfactory cryo-protectants.

(b) Re-establishment of the thawed stabilates require certain minimum rates of seed cells. These differ from one strain to another.

(c) The most satisfactory containers used for low temperature storage were ampoules and tightly sealed Bijou bottles.

(d) A storage temperature of -24°C was unsatisfactory for infected cells but when these were taken from dry ice and liquid nitrogen storage and re-stored at -24°C, the schizont-infected cells survived over a seven-day period.

(e) A storage temperature of -78°C was satisfactory for preservation of schizont-infected cells for a few months but, for longer storage, -150°C was preferable.

(f) For the establishment of a culture after low temperature storage a certain minimal percentage viability is essential irrespective of total numbers of cells.

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REFERENCES

Adler, S. and Ellenbogen, V. (1935). Observation on theileriosis in Palestine. Archs. Inst. Pasteur Algér., 13, 451-488.

Axelrad, A.A. and McCulloch, E.A. (1958). Obtaining suspensions of animal cells in metaphase from culture propagated on glass. Stain Technol., 33, 67-71.

Barnett, S.F. and Brocklesby, D.W. (1966a). Recent investigations into theileridae of cattle and buffalo in Africa. A mild form of East Coast fever (Theileria parva) with persistence of infection. Br. vet. J., 122, 361-370.

Barnett, S.F. and Brocklesby, D.W. (1966b). The susceptibility of the African buffalo (Syncerus caffer) to infection with Theileria parva (Theiler, 1904). Br. vet. J., 122, 379-386.

Begemann, H. and Rastetter, J. (1972). "Atlas of clinical haematology". Second edition, Springer-Verlag, Berlin. Heidleberg. New York.

Biggers, J.D. and McFeely, R.A. (1963). A simple method for the display of chromosomes from cultures of white blood cells with special reference to the ox. Nature, Lond., 199, 718-719.

Böyum, A. (1964). Separation of white blood cells. Nature, Lond., 204, 793.

Böyum, A. (1968). Isolation of leucocytes from human blood. Further observation. Scand. J. clin. Lab. Invest., 21, 31-50.

Brocklesby, D.W. (1956). Attempts to grow T. parva, the causal organism of East Coast fever, in tissue culture. Ann. Rep. EAVRO., 29-33.

Brocklesby, D.W. (1970). The development of Theileria parva in bovine tissues. J. Parasit., Second int. Congress, Washington, D.C., 35-36.

Brocklesby, D.W. and Barnett, S.F. (1966a). The literature concerning theileridae of the African buffalo (Syncerus caffer). Br. vet. J., 122, 371-378.

Brocklesby, D.W. and Barnett, S.F. (1966b). The isolation of Theileria lawrencei (Kenya) from a wild buffalo (Syncerus caffer) and its serial passage through captive buffaloes. Br. vet. J., 122, 387-395.

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. Br. vet. J., 117, 529-531.

Brocklesby, D.W. and Hawking, F. (1958). Growth of Theileria annulata and T. parva in tissue culture. Trans. R. Soc. trop. Med. Hyg., 52, 414-420.

Buckland, R. (1974). Chromosome preparation from bovine peripheral blood. Personal communication. (MRC, Cytogenetic Unit, Western General Hospital, Edinburgh).

Busby, D.W.G., House, W. and Macdonald, J.R. (1964). Sub-zero cabinet. In: "Virology technique". J. and A. Churchill Ltd., London, 33-34.

Carlson, G.P. and Kaneko, J.J. (1973). Isolation of leucocytes from bovine peripheral blood. Proc. Soc. exp. Biol. Med., 142, 853-856.

Cowdry, E.V. and Ham, A.W. (1932). Studies on East Coast fever. I. The life-cycle of the parasite in ticks. Parasitology, 24, 1-49.

Cunningham, M.P., Brown, C.G.D., Burridge, M.J. and Purnell, R.E. (1973). Cryopreservation of infective particles of Theileria parva. Int. J. Parasit., 3, 583-587.

Cunningham, M.P., Lumsden, W.H.R. and Webber, W.A.F. (1963). Preservation of viable trypanosomes in lymph tubes at low temperature. Expl. Parasit., 14, 280-284.

Dacie, J.V. and Lewis, S.M. (1970). "Practical Haematology". Fourth edition, J. and A. Churchill, London.

Dalgliesh, R.J. (1972). Theoretical and practical aspects of freezing parasitic protozoa. Aust. vet. J., 48, 233-239.

Danskin, D. and Wilde, J.K.H. (1976a). Simulation in vitro of bovine host cycle of Theileria parva. Nature, Lond., 261, 311-312.

Danskin, D. and Wilde, J.K.H. (1976b). The effect of calf lymph and bovine red blood cells on in vitro cultivation of Theileria parva-infected lymphoid cells. Trop. Anim. Hlth Prod., 8, 175-185.

DeKoch, 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 J. vet. Res., 27, 431-452.

Diamond, L.S. (1964). Freeze-preservation of protozoa. Cryobiology, 1, 95-102.

Donatien, A. and Lestoquard, F. (1938). Sur quelques maladies subtropicales du méditerranéen. Archs. Inst. Pasteur Algér., 17, 322-330.

Dschunkowsky, E. and Luhs, J. (1904). Die piroplasmosen der Rinder (Vorl. Mitt.). Cbl. f. Bakt. 1 Abt., Org., 35, 486-492.

Eagle, H. (1955a). The minimum vitamin requirements of the L and He La Cells in tissue culture, the production of specific vitamin deficiencies and their cure. J. exp. Med., 102, 595-600.

Eagle, H. (1955b). The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. J. biol. Chem., 214, 839-852.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. Science, N.Y., 130, 432-437.

Favour, C.B. (1964). Antigen-antibody reactions in tissue culture. In: "Immunological methods". J.F. Ackroyd (ed.). Blackwell Scientific Publications, Oxford, 195-223.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S. and Singh, R.M.M. (1966). Hydrogen ion buffers for biological research. Biochemistry, 5, 467-477.

Harris, R. and Ukaejiofo, E.V. (1969). Rapid preparation for lymphocytes for tissue typing. Lancet, 2, 327.

Hashemi-Fesharki, R. and Shad-Del, F. (1973a). Long term maintenance of Theileria annulata strains by freezing at -70°C. Vet. Rec., 92, 150-151.

Hashemi-Fesharki, R. and Shad-Del, F. (1973b). Vaccination of calves and milking cows with different strains of Theileria annulata. Am. J. vet. Res., 34, 1465-1467.

Hashemi-Fesharki, R. and Shad-Del, F. (1974). The therapeutic value of oxytetracycline hydrochloride (terramycin) in cattle infected experimentally with Theileria annulata. Trop. Anim. Hlth Prod., 6, 119-121.

Hawking, F. (1945). Growth of protozoa in tissue culture. I. Plasmodium gallinaceum, exoerythrocytic forms. Trans. R. Soc. trop. Med. Hyg., 39, 245-263.

Hawking, F. (1951). Tissue culture of plasmodia. Br. med. Bull., 8, 16-21.

Hawking, F. (1958). Chemotherapeutic screening of compounds against Theileria annulata in tissue culture. Br. J. Pharmac., 13, 458-460.

Henry, R.J. (1964). "Clinical Chemistry, principles and technics". Hoeber Medical Division, Harper and Row, Publishers, N.Y.

Hooshmand-Rad, P. (1973). "Some studies on Theileria annulata with special reference to an attenuated vaccine". Ph.D. Thesis, University of Edinburgh.

Hooshmand-Rad, P. (1975). The growth of Theileria annulata infected cells in suspension culture. Trop. Anim. Hlth Prod., 7, 23-28.

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. Archs. Inst. Razi, (Hessarak), 20, 85-89.

Hulliger, L. (1965). Cultivation of three species of Theileria in lymphoid cells in vitro. J. Protozool., 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, Lond., 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, Lond., 203, 728-730.

Hungerford, D.A., Donnelly, A.J., Nowell, P.C. and Beck, S. (1959). The chromosome constitution of a human phenotypic intersex. Am. J. hum. Genet., 11, 215.

Irvin, A.D., Brown, C.G.D., Boarer, C.D.H., Crawford, J.G. and Kanhai, G.K. (1974). Autoradiographic evidence for the occurrence of cell fusion in cultures of Theileria-infected bovine lymphoid cells. Res. vet. Sci., 16, 137-142.

Jacoby, F. (1944). A method of obtaining permanent preparations for the cytological study of pure cultures of macrophages, with special reference to their mode of division. J. Path., 56, 438-442.

Jarret, W.F.H. and Brocklesby, D.W. (1966). A preliminary microscopic study of East African fever (Theileria parva infection). J. Protozool., 13, 301-310.

Jarret, W.F.H., Crighton, G.W. and Pirie, H.M. (1969). Theileria parva: Kinetic of replication. Expl. Parasit., 24, 9-25.

Joel, D.D., Adamik, E.R., Chanana, A.D., Cronkite, E.P., Sciffer, L.M. and Sipe, C.R. (1969). Separation of lymphocytes from blood of calves and goats. Am. J. vet. Res., 30, 1099-1104.

Joyner, L.P. and Brocklesby, D.W. (1973). Chemotherapy of anaplasmosis, babesiosis and theileriosis. Adv. in Pharmacol. Chemother., 11, 321-355.

Knight, S.C. (1967). "The responses of mammalian lymphocytes to stimulation." Ph.D. Thesis, University of Birmingham.

Knight, S.C., Bradley, J., Oppenheim, J.J. and Ling, N.R. (1968). The in vitro transformation of thymocytes and lymphocytes from humans, rabbits and guinea-pigs and from thymomas. Clin. exp. Immunol., 3, 323-341.

Knight, S., Ling, N.R., Sell, S. and Oxnard, C.E. (1965). The transformation in vitro of peripheral lymphocytes of some laboratory animals. Immunology, 9, 565-574.

Kosenow, V.W. and Pfeiffer, R.A. (1961). Chromosomendiagnostik in blut-und knochenmarkzellen. Blut, 7, 500-508.

Leibovitz, A. (1963). The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. Am. J. Hyg., 78, 173-180.

Levine, D.N. (1973). "Protozoan parasites of domestic animals and of man". Second edition. Burgess Publishing Company. Minneapolis, Minnesota, 26-32.

Li, I.G. and Osgood, E.E. (1949). A method for the rapid separation of leucocytes and nucleated erythrocytes from blood or marrow with phytohaemagglutinin from red beans (Phaseolus vulgaris). Blood, 4, 670.

Ling, N.R. and Kay, J.E. (1975). "Lymphocyte stimulation". Revised edition. North-Holland Publishing Company-Amsterdam. Oxford.

Lovelock, J.E. and Bishop, M.W.H. (1959). Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature, Lond., 183, 1394-1395.

Malmquist, W.A. and Brown, C.G.D. (1974). Establishment of Theileria parva-infected lymphoblastoid cell lines using homologous feeder layers. Res. vet. Sci., 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. Trop. Anim. Hlth Prod., 2, 139-145.

Malmquist, W.A., Van Der Maaten, M.J. and Boothe, A.D. (1969). Isolation, immunodiffusion, immunofluorescence and electron microscopy of syncytial virus of lymphosarcomatous and apparently normal cattle. Cancer Res., 29, 188-200.

Mazur, P. (1970). Cryobiology: The freezing of biological systems. Science, N.Y., 168, 939-949.

Meryman, H.T. (1968). I. Preservation by freezing. In: "Infectious blood diseases of man and animals". Weinman, D. and Ristic, M. (eds.) Academic Press, New York and London, Vol.I, 343-353.

McHardy, N., Haigh, A.J.B. and Dolan, T.T. (1976). Chemotherapy of Theileria parva infection. Nature, Lond., 261, 698-699.

Moorhead, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M. and Hungerford, D.A. (1960). Chromosome preparations of leucocytes cultured from human peripheral blood. Expl. Cell Res., 20, 613-616.

Morgan, J.F., Morton, H.J. and Parker, R.C. (1950). Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. (17557), proc. Soc. exp. Biol. Med., 73, 1-8.

Moulton, J.E., Krauss, H.H. and Malmquist, W.A. (1971a). Growth characteristics of Theileria parva-infected bovine lymphoblast cultures. Am. J. vet. Res., 32, 1365-1370.

Moulton, J.E., Krauss, H.H. and Malmquist, W.A. (1971b). Transformation of reticulum cells to lymphoblasts in cultures of bovine spleen infected with Theileria parva. Lab. Invest., 24, 187-196.

Neitz, W.O. (1957). Theileriosis, Gonderioses and Cytauxzoones: A review. Onderstepoort J. vet. Res., 27, 275-430.

Nichols, W.W., Levan, A. and Lawrence, W.C. (1962). Bovine chromosomes by the peripheral blood method. Hereditas, 48, 536-538.

Nowell, P.C. (1960). Phytohaemagglutinin: an initiator of mitosis in cultures of normal human leucocytes. Cancer Res., 20, 462.

Odajima, M. and Sonoda, M. (1970). Haematological observations of leucocytes collected by water method from bovine peripheral blood. Jap. J. vet. Res., 18, 173-179.

Odajima, M. and Sonoda, M. (1971). Haematological observations of leucocytes collected from bovine peripheral blood by an improved ammonium chloride method. Jap. J. vet. Sci., 33, 295-300.

Paul, J. (1975). "Cell and tissue culture". Fifth edition, Churchill Livingstone, Edinburgh, London and New York.

Pegg, D.E. (1976). Long-term preservation of cells and tissues: a review. J. clin. Path., 29, 271-285.

Pipano, E. (1965). Piroplasmosis - A review, Theileria. Refuah vet., 22, 175-181.

Pipano, E. and Tsur, I. (1966). Experimental immunization against Theileria annulata with a tissue culture vaccine. I. Laboratory trials. Refuah vet., 23, 186-194.

Polge, C., Smith,A.U. and Parkes, A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperature. Nature, Lond., 164, 666.

Polge, D.E. and Soltys, M.A. (1957). Preservation of trypanosomes in the frozen state. Trans. R. Soc. trop. Med. Hyg., 51, 519-526.

Rafyi, A., Maghami, G. and Hooshmand-Rad (Houshmand), P. (1965). Sur la virulence de Theileria annulata (Dschunkowsky et Luhs 1904) et prèmunition contre theilériose bovines en Iran. Bull. Off. int. Épizoot., 64, 431-446.

Rafyi, A., Maghami, G. and Hooshmand-Rad (Houshmand), P. (1967). Studies on the antigenic value and premunition against bovine theileriosis due to *Theileria annulata* (Dschunkowsky and Luhs 1904) in Iran and conservation of strains of *Theileria annulata* at -70°C. Bull. Off. int. Épizoot., 68, 749-755.

Reichenow, E. (1940). Der Entwicklungsgang des Kustenfiebererreges in Rinde und in der übertragenden Zecke. Arch. Protistenk., 94, 1-56.

Reichenow, E. (1941). Zur Kenntnis des Kustenfiebers der Rinder. Dt. tierärztl. Wschr., 49, 546-547 and 594-595.

Rothfels, K.H. and Siminovitch, L. (1958). An air-drying technique for flattening chromosomes in mammalian cells grown *in vitro*. Stain Technol., 33, 73-77.

Roos, D. and Loos, J.A. (1970). Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. I. Stimulation by phytohaemagglutinin. Biochim. biophys. Acta, 222, 565-582.

Rowe, A.W. (1970). Storage of lymphocytes in the frozen state. In: "A Seminar on Histocompatibility Testing". The American Association of blood Banks, 47-58.

Sasaki, M.S. and Makino, S. (1962). Revised study of the chromosomes of domestic cattle and the horse. J. Hered., 53, 157-162.

Schalm, O.W., Jain, N.C. and Carroll, E.J. (1975). "Veterinary Hematology". Third edition. Lea and Febiger, Philadelphia.

Sergent, E., Donatien, A., Parrot, L. and Lestoquard, F. (1936a). Cycle évolutif du sporozaire *Theileria dispar*, agent de la thelériose bovine des pays méditerranéens, chez le boeuf et chez une tique. C.r. hebd. Séanc. Acad. Sci., Paris, 202 and 809.

Sergent, E., Donatien, A., Parrot, L. and Lestoquard, F. (1936b). Étude morphologique du cycle évolutif de *Theileria dispar* chez le boeuf et chez la tique. Ann. Inst. Pasteur, 57, 30-55.

Sergent, E., Donatien, A., Parrot, L. and Lestoquard, F. (1936c). Cycle évolutif de *Theileria dispar*, du boeuf, chez la tique *Hyalomma mauritanicum*. Archs. Inst. Pasteur Algér., 14, 259-294.

Sergent, E., Donatien, A., Parrot, L. and Lestoquard, F. (1945). Étude sur les piroplasmoses bovines. Alger: Inst. Pasteur d'Algérie, 816.

Sergent, E., Donatien, A., Parrot, L., Lestoquard, F., Plantureux, E. and Rougebief, H. (1924a). Les piroplasmoses bovines d'Algérie. Premier mémoire. Archs. Inst. Pasteur Algér., 2, 1-146.

Sergent, E., Donatien, A., Parrot, L., Lestoquard, F., Plantureux, E. and Rougebief, H. (1924b). Études expérimentales sur les piroplasmoses bovines d'Algérie. Ann. Inst. Pasteur, 38, 273-344.

Shad-Del, F. (1973a). Vaccination of calves and milking cows of susceptible exotic breeds with a virulent strain of Theileria annulata, S.3 attenuated in tissue culture. Unpublished data.

Shad-Del, F. (1973b). Retrieval of deep-frozen schizonts of Theileria annulata. Unpublished data.

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). Res. vet. Sci., 16, 125-127.

Stagg, D.A., Kanhai, G.K., Young, A.S. and Brown, C.G.D. (1976). The establishment of Theileria-infected cell lines from an eland (Taurotragus oryx, Lydekker 1906). Res. vet. Sci., 20, 122-126.

Stecher, P.G., Windholz, M., Leahy, D.S., Bolton, M. and Eaton, L.G. (1968). "The Merk Index, an encyclopedia of chemicals and drugs". Eighth edition. Merk and Co. Inc. Rahway, N.J., U.S.A., 378 and 499.

Taylor, B.J. (1975). "Quantitative studies on the cryopreservation of piroplasms with particular reference to Babesia rodhaini". Ph.D. Thesis, University of Edinburgh.

Thorsby, E. and Bratlie, A. (1970). A rapid method for preparation of pure lymphocyte suspensions. In: "Histocompatibility testing". Terasaki, P.I. (ed.), Munksgaard, Copenhagen, 655.

Ting, A. and Morris, P.J. (1971). A technique for lymphocyte preparation from stored heparinised blood. Vox. Sang., 20, 561.

Tokuda, G., Fukusho, K., Morimoto, T. and Watanabe, M. (1962). Studies on rinderpest virus in bovine leucocyte culture. I. Cultivation of leucocytes and appearance of inclusions in infected cells. Nat. Inst. Anim. Hlth Quart., 2, 189-200.

Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. clin. Biochem., 6, 24-27.

Tsur (Tchernomoretz), I. (1945). Multiplication in vitro of Koch bodies of Theileria annulata. Nature, Lond., 156, 391.

Tsur (Tchernomoretz), I. (1947). Multiplication in vitro of Koch bodies of Theileria annulata. Refuah vet., 4, 86.

Tsur (Tchernomoretz), I. (1953). Theileria annulata et Leishmania en culture de tissu. 15th int. vet. Congress, Stockholm, 1, 26-31.

Tsur, I. and Adler, S. (1962). Cultivation of Theileria annulata schizonts in monolayer tissue cultures. Refuah vet., 19, 224-225.

Tsur, I. and Adler, S. (1965). The cultivation of lymphoid cells and Theileria annulata schizonts from infected bovine blood. Refuah vet., 22, 60-62.

Tsur, I., Adler, S., Pipano, E. and Senft, Z. (1964). Continuous growth of Theileria annulata schizonts in monolayer tissue cultures. Proc. first int. Congress Parasitol., Rome, 1, 266-267.

Tsur, I., Neitz, W.O. and Pols, J.W. (1957). The development of Koch bodies of Theileria parva in tissue culture. Refuah vet., 14, 51-53.

Tsur-Tchernomoretz, I. and Pipano, E. (1959). Growth and multiplication of Koch bodies of Theileria annulata in rodents' spleen tissue culture. Refuah vet., 16, 51.

Tsur, I. and Pipano, E. (1962). The successful infection of cattle with Theileria annulata parasites preserved in the frozen state. Refuah vet., 19, 110.

Tsur, I. and Pipano, E. (1966). Attenuation of T. annulata by growth and passage through tissue culture. J. Protozool., 13, 33-34.

Ulbrich, F., Weinhold, E. and Pfeiffer, R.A. (1963). Preparation of bovine chromosomes. Nature, Lond., 199, 719.

Weir, D.M. (1974). "Handbook of experimental immunology". Second edition. Blackwell Scientific Publications, Oxford, London, Edinburgh and Melbourne.

Wilde, J.K.H. (1966). Changes in bovine marrow during the course of East Coast fever. Res. vet. Sci., 7, 213-224.

Wilde, J.K.H. (1967). East Coast fever. Adv. vet. Sci., 11, 207-259.

Wilde, J.K.H. (1975). Theileriasis in East Africa. Proc. 20th World vet. Congress, Thessaloniki, Greece, 481-486.

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. Br. vet. J., 124, 196-208.

Wilde, J.K.H., Hulliger, L. and Brown, C.G.D. (1966). Some recent East Coast fever research. Bull. epizoot. Dis. Afr., 14, 29-35.

Zablotskyi, V.T. (1966). Cultivation of Theileria annulata schizonts in cultures of bovine lymph nodes and spleen. Mater. godichnoi nauchoi Konf. Viev. 70-72. (Vet. Bull., Weybridge, Abst. No. 2651, 38, 1968).

Zablotskyi, V.T. (1967). Use of tissue culture in the study of Theileria annulata. Veterinariya, 44, 66-69 (Vet. Bull., Weybridge, Abst. No. 2652, 38, 1968).

# APPENDIX TABLES

Appendix Table 1

Giemsa stain

| | |
|---|---|
| Azur II eosin | 3.0g |
| Azur II | 0.8g |
| Glycerol | 250ml |
| Pure methanol | 250ml |

Instead of azur II eosin and azur II, 3.8g of the compounded Giemsa stain powder may be used. Place all in a flask, plug neck loosely with cotton wool and warm on a water bath for one hour; cool and filter it.

Appendix Table 2

May-Grünwald/Giemsa stain

Solutions: (A) May-Grünwald stain

(B) Giemsa stain

Technique:

1. Unfixed air dried films are stained for one minute in May-Grünwald stain, then an equal volume of distilled water is added and mixed with the stain by rocking the slide. The diluted stain is allowed to act for one minute, then drained off without washing.

2. Stain for ten to fifteen minutes in a mixture consisting of ten drops of Giemsa stain in ten ml distilled water.

3. Differentiate for about five seconds with distilled water, examining under the microscope to ensure that differentiation is complete.

4. Blot, dry in air and mount if desired.

Appendix Table 4

Difference of growth rates of lymphoid cells infected with various strains of T. annulata cultivated in modified Eagle's medium supplemented with CS and LY (Experiment 5.1).

| Strain | Culture No. | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| S.3 | 1 | 100.0 | 188.0 | 394.0 | 490.0 | 560.0 | 495.0 |
| | 2 | 100.0 | 130.0 | 305.0 | 486.0 | 465.0 | 410.0 |
| | 3 | 100.0 | 157.0 | 320.0 | 515.0 | 580.0 | 472.0 |
| | M. | | 158.3 | 339.7 | 497.0 | 535.0 | 459.0 |
| | S.D. | | 29.0 | 47.6 | 15.7 | 61.4 | 43.9 |
| S.15 | 1 | 100.0 | 212.0 | 405.0 | 653.0 | 1045.0 | 883.0 |
| | 2 | 100.0 | 135.0 | 310.0 | 596.0 | 1015.0 | 915.0 |
| | 3 | 100.0 | 188.0 | 354.0 | 615.0 | 990.0 | 867.0 |
| | M. | | 178.3 | 356.3 | 621.3 | 1016.7 | 888.3 |
| | S.D. | | 39.4 | 47.5 | 29.0 | 27.5 | 24.4 |
| S.19 | 1 | 100.0 | 111.0 | 233.0 | 440.0 | 638.0 | 505.0 |
| | 2 | 100.0 | 88.0 | 205.0 | 370.0 | 486.0 | 535.0 |
| | 3 | 100.0 | 123.0 | 230.0 | 412.0 | 520.0 | 453.0 |
| | M. | | 107.3 | 222.7 | 407.3 | 548.0 | 497.7 |
| | S.D. | | 17.8 | 15.4 | 35.2 | 79.8 | 41.5 |
| S.20 | 1 | 100.0 | 165.0 | 374.0 | 618.0 | 742.0 | 690.0 |
| | 2 | 100.0 | 130.0 | 280.0 | 465.0 | 664.0 | 585.0 |
| | 3 | 100.0 | 148.0 | 315.0 | 504.0 | 700.0 | 624.0 |
| | M. | | 147.7 | 323.0 | 529.0 | 702.0 | 633.0 |
| | S.D. | | 17.5 | 47.5 | 79.5 | 39.0 | 53.1 |
| S.21 | 1 | 100.0 | 194.0 | 404.0 | 597.0 | 820.0 | 690.0 |
| | 2 | 100.0 | 152.0 | 335.0 | 475.0 | 715.0 | 638.0 |
| | 3 | 100.0 | 163.0 | 350.0 | 604.0 | 745.0 | 586.0 |
| | M. | | 169.7 | 363.0 | 558.7 | 760.0 | 638.0 |
| | S.D. | | 21.8 | 36.3 | 72.5 | 54.1 | 52.0 |

No. of viable cells per ml, counted daily from each strain, x $10^3$

M. = Mean

S.D. = Standard Deviation

Appendix Table 5

Attempts to cultivate schizont-infected cells in some simple growth media compared with modified Eagle's medium, control (Experiment 5.2).

| Media | Trial | Culture | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 |
| (a) PBS + CS + LY | 1 | 1 | 100.0 | 120.0 | 175.0 | 130.0 | 105.0 | |
| | | 2 | 100.0 | 95.0 | 165.0 | 110.0 | 45.0 | |
| | | 3 | 100.0 | 70.0 | 95.0 | 35.0 | 10.0 | |
| | 2 | 1 | 100.0 | 112.0 | 150.0 | 75.0 | 60.0 | |
| | | 2 | 100.0 | 130.0 | 80.0 | 60.0 | 40.0 | |
| | | 3 | 100.0 | 85.0 | 65.0 | 40.0 | 15.0 | |
| | 3 | 1 | 100.0 | 95.0 | 170.0 | 115.0 | 95.0 | |
| | | 2 | 100.0 | 120.0 | 75.0 | 50.0 | 25.0 | |
| | | 3 | 100.0 | 35.0 | 5.0 | 0.0 | 0.0 | |
| Control | | | 100.0 | 155.0 | 370.0 | 680.0 | 1005.0 | 890.0 |
| (b) Hank's BSS + CS + LY | 1 | 1 | 100.0 | 80.0 | 125.0 | 65.0 | 55.0 | |
| | | 2 | 100.0 | 110.0 | 175.0 | 85.0 | 35.0 | |
| | | 3 | 100.0 | 90.0 | 70.0 | 30.0 | 10.0 | |
| | 2 | 1 | 100.0 | 135.0 | 160.0 | 80.0 | 50.0 | |
| | | 2 | 100.0 | 120.0 | 120.0 | 70.0 | 40.0 | |
| | | 3 | 100.0 | 90.0 | 115.0 | 80.0 | 35.0 | |
| | | 4 | 100.0 | 75.0 | 50.0 | 10.0 | 0.0 | |
| | 3 | 1 | 100.0 | 90.0 | 125.0 | 85.0 | 60.0 | |
| | | 2 | 100.0 | 105.0 | 115.0 | 60.0 | 25.0 | |
| | | 3 | 100.0 | 75.0 | 90.0 | 45.0 | 20.0 | |
| | | 4 | 100.0 | 70.0 | 35.0 | 0.0 | 0.0 | |
| Control | | | 100.0 | 130.0 | 325.0 | 650.0 | 955.0 | 820.0 |
| (c) Earle's BSS + CS + LY | 1 | 1 | 100.0 | 145.0 | 190.0 | 125.0 | 105.0 | |
| | | 2 | 100.0 | 115.0 | 150.0 | 105.0 | 60.0 | |
| | | 3 | 100.0 | 105.0 | 80.0 | 40.0 | 20.0 | |
| | | 4 | 100.0 | 120.0 | 95.0 | 40.0 | 15.0 | |
| | 2 | 1 | 100.0 | 110.0 | 185.0 | 130.0 | 95.0 | |
| | | 2 | 100.0 | 95.0 | 120.0 | 70.0 | 35.0 | |
| | | 3 | 100.0 | 85.0 | 70.0 | 25.0 | 0.0 | |
| | 3 | 1 | 100.0 | 165.0 | 210.0 | 140.0 | 70.0 | |
| | | 2 | 100.0 | 125.0 | 105.0 | 80.0 | 40.0 | |
| | | 3 | 100.0 | 130.0 | 105.0 | 70.0 | 20.0 | |
| | | 4 | 100.0 | 65.0 | 20.0 | 0.0 | 0.0 | |
| Control | | | 100.0 | 180.0 | 405.0 | 715.0 | 900.0 | 765.0 |

No. of cells per ml, x $10^3$

Appendix Table 6

Comparison of growth rate of schizont-infected cells cultivated in various standard media, all supplemented with CS and LY (Experiment 5.3).

| Media | Culture | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| (a) Modified Eagle's | 1 | 100.0 | 125.0 | 300.0 | 595.0 | 1095.0 | 930.0 |
| | 2 | 100.0 | 160.0 | 375.0 | 660.0 | 1160.0 | 990.0 |
| | 3 | 100.0 | 90.0 | 270.0 | 540.0 | 1040.0 | 855.0 |
| | Mean | | 125.0 | 315.0 | 598.3 | 1098.3 | 925.0 |
| | S.D. | | 35.0 | 54.1 | 60.1 | 60.1 | 67.6 |
| (b) TC medium 199 | 1 | 100.0 | 115.0 | 325.0 | 570.0 | 955.0 | 880.0 |
| | 2 | 100.0 | 135.0 | 340.0 | 620.0 | 1020.0 | 935.0 |
| | 3 | 100.0 | 90.0 | 230.0 | 490.0 | 865.0 | 800.0 |
| | Mean | | 113.3 | 298.3 | 560.0 | 946.7 | 871.7 |
| | S.D. | | 22.6 | 59.7 | 65.6 | 77.8 | 67.9 |
| (c) Eagle's MEM + Hank's BSS | 1 | 100.0 | 190.0 | 415.0 | 765.0 | 1605.0 | 1225.0 |
| | 2 | 100.0 | 210.0 | 490.0 | 800.0 | 1865.0 | 1340.0 |
| | 3 | 100.0 | 150.0 | 325.0 | 625.0 | 1410.0 | 1060.0 |
| | Mean | | 183.3 | 410.0 | 730.0 | 1626.7 | 1208.3 |
| | S.D. | | 30.6 | 82.6 | 92.6 | 228.3 | 140.7 |
| (d) Eagle's MEM + Earle's BSS | 1 | 100.0 | 140.0 | 320.0 | 650.0 | 1525.0 | 1380.0 |
| | 2 | 100.0 | 155.0 | 380.0 | 735.0 | 1820.0 | 1450.0 |
| | 3 | 100.0 | 115.0 | 250.0 | 620.0 | 1315.0 | 1140.0 |
| | Mean | | 136.7 | 316.7 | 668.3 | 1553.3 | 1323.3 |
| | S.D. | | 20.2 | 65.1 | 59.7 | 253.7 | 162.6 |

No. of cells per ml, x $10^3$

S.D. = Standard Deviation

Appendix Table 7

Growth rate of schizont-infected cells cultivated in Eagle's MEM with Hank's salts and CS and with different supplements (Experiment 5.4).

| Supplements | Culture | Days of cultivation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| (a) LY | 1 | 100.0 | 135.0 | 265.0 | 710.0 | 1545.0 | 1205.0 |
| | 2 | 100.0 | 145.0 | 315.0 | 625.0 | 1380.0 | 1145.0 |
| | 3 | 100.0 | 160.0 | 310.0 | 735.0 | 1765.0 | 1335.0 |
| | Mean | | 146.7 | 296.7 | 690.0 | 1563.3 | 1228.3 |
| | S.D. | | 12.6 | 27.5 | 57.7 | 193.2 | 97.1 |
| (b) TPB | 1 | 100.0 | 140.0 | 225.0 | 575.0 | 1120.0 | 905.0 |
| | 2 | 100.0 | 125.0 | 360.0 | 565.0 | 955.0 | 880.0 |
| | 3 | 100.0 | 150.0 | 390.0 | 505.0 | 730.0 | 710.0 |
| | Mean | | 138.3 | 325.0 | 548.3 | 935.0 | 831.7 |
| | S.D. | | 12.6 | 87.9 | 37.9 | 195.8 | 106.1 |
| (c) L-β-asparagine | 1 | 100.0 | 155.0 | 335.0 | 515.0 | 990.0 | 840.0 |
| | 2 | 100.0 | 115.0 | 220.0 | 395.0 | 850.0 | 730.0 |
| | 3 | 100.0 | 85.0 | 190.0 | 370.0 | 720.0 | 680.0 |
| | Mean | | 118.3 | 248.3 | 426.7 | 853.3 | 750.0 |
| | S.D. | | 35.1 | 76.5 | 77.5 | 135.0 | 81.8 |
| (d) NEAA | 1 | 100.0 | 150.0 | 295.0 | 545.0 | 1250.0 | 1110.0 |
| | 2 | 100.0 | 130.0 | 220.0 | 400.0 | 960.0 | 900.0 |
| | 3 | 100.0 | 75.0 | 180.0 | 305.0 | 840.0 | 810.0 |
| | Mean | | 118.3 | 231.7 | 416.7 | 1016.7 | 940.0 |
| | S.D. | | 38.8 | 58.4 | 120.9 | 210.8 | 153.9 |
| (e) Control medium, without supplement | 1 | 100.0 | 125.0 | 180.0 | 370.0 | 820.0 | 580.0 |
| | 2 | 100.0 | 80.0 | 135.0 | 295.0 | 675.0 | 565.0 |
| | 3 | 100.0 | 110.0 | 160.0 | 380.0 | 830.0 | 460.0 |
| | Mean | | 105.0 | 158.3 | 348.3 | 775.0 | 535.0 |
| | S.D. | | 22.9 | 22.5 | 46.5 | 86.7 | 65.4 |

No. of cells per ml, x $10^3$

S.D. = Standard Deviation

Appendix Table 8

Effect of pH of the medium on growth rate of schizont-infected cells (Experiment 5.5).

| Buffer | Culture | Days of cultivation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (a) Sodium bicarbonate | 1 | 50.0 | 45.0 | 110.0 | 320.0 | 635.0 | 1245.0 | 905.0 | 555.0 |
| | 2 | 50.0 | 95.0 | 165.0 | 365.0 | 670.0 | 1460.0 | 875.0 | 505.0 |
| | 3 | 50.0 | 40.0 | 130.0 | 290.0 | 560.0 | 965.0 | 705.0 | 435.0 |
| | 4 | 50.0 | 85.0 | 150.0 | 460.0 | 890.0 | 1430.0 | 930.0 | 575.0 |
| | Mean | | 66.2 | 138.7 | 358.7 | 688.7 | 1275.0 | 853.7 | 517.5 |
| | S.D. | | 27.8 | 23.9 | 74.2 | 141.8 | 227.5 | 101.7 | 62.4 |
| (b) Sodium bicarbonate + $CO_2$ | 1 | 50.0 | 110.0 | 255.0 | 760.0 | 1560.0 | 1225.0 | 840.0 | 605.0 |
| | 2 | 50.0 | 90.0 | 205.0 | 590.0 | 1310.0 | 1105.0 | 835.0 | 635.0 |
| | 3 | 50.0 | 80.0 | 175.0 | 425.0 | 1405.0 | 1215.0 | 860.0 | 595.0 |
| | 4 | 50.0 | 65.0 | 145.0 | 405.0 | 1370.0 | 1460.0 | 1105.0 | 910.0 |
| | Mean | | 86.2 | 195.0 | 545.0 | 1411.2 | 1251.2 | 910.0 | 686.2 |
| | S.D. | | 18.9 | 46.9 | 165.6 | 106.6 | 149.4 | 130.4 | 150.1 |
| (c) Sodium bicarbonate + Hepes | 1 | 50.0 | 75.0 | 145.0 | 295.0 | 730.0 | 1520.0 | 1290.0 | 1045.0 |
| | 2 | 50.0 | 115.0 | 235.0 | 460.0 | 855.0 | 1640.0 | 1760.0 | 1275.0 |
| | 3 | 50.0 | 95.0 | 210.0 | 250.0 | 725.0 | 1555.0 | 1230.0 | 1060.0 |
| | 4 | 50.0 | 110.0 | 235.0 | 485.0 | 870.0 | 1615.0 | 1525.0 | 1120.0 |
| | Mean | | 98.7 | 206.2 | 372.5 | 795.0 | 1582.5 | 1451.2 | 1125.0 |
| | S.D. | | 18.0 | 42.5 | 117.4 | 78.2 | 54.8 | 242.0 | 105.1 |

No. of cells per ml, x $10^3$

S.D. = Standard Deviation

## Appendix 9

Method used to count cells in suspension culture.

### (a) Sampling

The culture vessel on all occasions was rocked in a uniform manner five times. By means of a pipette the suspension was aspirated and ejected five times to make the cells evenly dispersed in the suspension. A sample of two ml was transferred to a non-wettable plastic tube. To this were now added one ml of a 0.2 per cent trypan blue solution and one ml of the medium containing 20 per cent calf serum. In this way the cell suspension was diluted two times, mixed thoroughly and allowed to stand for one minute. Afterwards the cell suspension was pipetted five times and then the appropriate amount was quickly transferred to one platform of the Improved Neubauer counting chamber. The cells were counted immediately using x20 objective. The whole process was repeated, the resultant suspension being transferred to the second platform of the counting chamber.

### (b) Counting

The cells in four, one millimeter squares occupying the corners of the grid were counted and the number per ml calculated from the formula:

$$\frac{X \times 10 \times 1000 \times 2}{4}$$

The mean of the duplicated counts was taken as the number of cells in the culture. In most cases the cells appeared to be evenly dispersed in the haemocytometer as shown in the example of the table of Appendix 9. When suspensions of non-infected cells were being counted, however, clumping was observed in some samples and the whole process was repeated until the clumps were broken

up and an even dispersal of cells was achieved.

| Count No. | No. of cells in one $mm^2$ of haemocytometer | day of sampling | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 1 | 5 | 15 | 38 | 55 | 70 | 44 |
| | 2 | 4 | 17 | 33 | 60 | 73 | 49 |
| | 3 | 4 | 14 | 35 | 54 | 69 | 40 |
| | 4 | 5 | 18 | 35 | 55 | 76 | 50 |
| | Total | 18 | 64 | 141 | 224 | 288 | 183 |
| 2 | 1 | 4 | 14 | 35 | 49 | 64 | 47 |
| | 2 | 4 | 15 | 37 | 54 | 60 | 45 |
| | 3 | 6 | 17 | 36 | 56 | 71 | 52 |
| | 4 | 4 | 16 | 39 | 47 | 73 | 55 |
| | Total | 18 | 62 | 147 | 206 | 268 | 199 |
| Mean of 2 counts | | 18 | 63 | 144 | 215 | 278 | 191 |
| No. of cells/ml x $10^3$ | | 90 | 315 | 720 | 1075 | 1390 | 955 |

Detailed figures are given for the counts in Experiment 5.11 and relate to the culture containing 62.59 per cent viable cells at the time of seeding as shown in Table 5.11 and Fig. 5.11.

Appendix 10

References not consulted in the original


Bass, C.C. and Johns, F.M. (1912). The cultivation of malarial *Plasmodia* (*Plasmodium vivax* and *Plasmodium falciparum*) *in vitro*. J. exp. Med., *16*, 567. Quoted by Hawking (1945).

Bettencourt, A., Franca, C., Borges, J. (1907). Un cas de piroplasmose bacilleforme chez le daim. Arg. R. Inst. Bact., Camara pestana, *1*, 341. Quoted by Neitz (1957).

Coggeshall, L.T. (1939). Preservation of viable malaria parasites in the frozen state. Proc. Soc. exp. Biol. Med., *42*, 499-501. Quoted by Taylor (1975).

Gavrilov, W., Bobkoff, G. and Laurencin, S. (1938). Essai de culture en tissue de "*Plasmodium gallinaceum*" (Brumpt). Ann. Soc. Belge méd. trop., *18*, 429. Quoted by Hawking (1945).

Hawking, F. (1944). Tissue culture of malaria parasites (*Plasmodium gallinaceum*). Lancet, *1*, 693. Quoted by Hawking (1945).

Henger, R. and Wolfson, F. (1939). Tissue culture studies of parasites in reticuloendothelial cells in birds infected with *Plasmodium*. Am. J. Hyg., Sect. C., *29*, 83. Quoted by Hawking (1945).

Koch, R. (1898). Reiseberichte über Rinderpest, Bubonenpest in India und Afrika, Teste und Surrakrankheit, Texasfieber, unsw. J. Springer, Berlin. Quoted by Neitz (1957).

Laveran, A. and Mesnil, F. (1904). "Trypanosomes et Trypanosomiases". Masson et Cie., Paris. Quoted by Taylor (1975).

MacHattie, C. (1935). Theileriosis of young calves in Baghdad dairies. Trans. R. Soc. trop. Med. and Hyg., *28*, 649-654. Quoted by Neitz (1957).

Pavlov, P. (1942). Das vorkommen von Theileriose in Macedonien. Dtsch. tierärztl. Wschr., *50*, 458-460. Quoted by Neitz (1957).

Sen, S.K. and Srinivasan, M.K. (1937). Theileriasis of cattle in India. Indian J. vet. Sci., *7*, 15-37. Quoted by Neitz (1957).

Trager, W. (1941). Studies on conditions affecting survival *in vitro* of malarial parasite (*Plasmodium lophurae*). J. exp. Med., *16*, 567. Quoted by Hawking (1945).

Trager, W., (1943). Further studies on survival and development *in vitro* of malarial parasite. J. exp. Med., 77, 411. Quoted by Hawking (1945).

Yakinoff, W.L., Gousseff, W.F. (1936). Zur Frage der Modifikation der virulenz der *Theileria annulata*. Zeitschr. f. Infektkr. d. Haustiere, 50, 65-70. Quoted by Neitz (1957).

Yousif, N.B. (1969). *Theileria annulata* infection in Friesian cattle imported to Iraq from Europe. Vet. Rec., 84, 360-363. Quoted by Hooshmand-Rad (1973).