# A LIGHT AND ELECTRON MICROSCOPE INVESTIGATION OF THE HOST-PARASITE RELATIONSHIP IN THE BRAINS OF MICE WITH CONGENITAL TOXOPLASMOSIS

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### ABSTRACT

Impregnation of the wall of intact tissue cysts in the brains of mice with congenital toxoplasmosis, with reduced silver salts and protargol silver suggested that it was composed, at least in part, of components derived from the neuronal cytoskeleton. Electron microscopy extended these observations and revealed that intact tissue cysts were separated from the extra-cellular compartment by a layer of neurofibrillae enclosed within the host cell membranes. Immunohistochemical staining confirmed that this layer contained neurofilament protein. Interior to this layer was a much convoluted parasitophorous vacuole membrane; exterior was the host cell membrane. In most cases, synaptic plates were noted on the outer plasma membrane. In no instance were tissue cysts observed either within neuroglial cells or in the absence of a host cell. Electron immunocytochemistry, using a rabbit polyclonal anti-Toxoplasma IgG as the primary layer in immunogold staining, revealed that Toxoplasma antigenwas widely distributed within the matrix of the cyst, being most concentrated in the proximity of the inner surface of the parasitophorous vacuole membrane. Relatively little Toxoplasma antigen was detected directly associated with cystozoites. Small amounts of antigen were detected directly associated with cystozoites, within host cell components exterior to the parasitophorous vacuole membrane and in the host neuropil, immediately adjacent to the tissue cyst.
The inter-relationship between inflammatory lesions associated with small vessels, in close proximity to intact tissue cysts, suggest that there may be 'migration' of these cells, in an ordered sequence, towards the Toxoplasma antigen secreted from the tissue cysts.

To Deborah

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CHAPTER 1

INTRODUCTION

## 1.1     TOXOPLASMA

There appear to be three distinct developmental stages in the natural history of <u>Toxoplasma</u>.

First, there are the coccidian stages which  occur only in the intestine of the domestic cat and other Felidae Hutchison <u>et al</u>.(1969,1970,1971); Frenkel <u>et al</u>.(1970); Piekarski and Witte, (1976); Sheffield and Melton, (1970). These consist of trophozoites, schizonts, and gametocytes (Figure 1); the developmental stages lead to the formation of the undifferentiated oocyst, which under suitable environmental influences become sporulated (Figure 2). Secondly, the proliferative form (Figure 3) or endozoite (Hoare, 1972).  This form of the parasite is often referred to as the tachyzoite (Frenkel, 1973).Thirdly, the tissue cyst (Figure 4) which contains organisms termed cystozoites (Hoare, 1972).  This intracystic form of the parasite is often referred to as the bradyzoite (Frenkel, 1973).

The terms endozoite and cystozoite are probably more appropriate than bradyzoite and tachyzoite; intracystic forms of <u>Isospora felis</u> do not multiply slowly or otherwise Dubey (1977) and since <u>Toxoplasma</u> is closely related to <u>Isospora felis</u> the description of "fast" dividing, or "slow" dividing organisms, may be misleading taxonomically. These difficulties in classification have been discussed by Levine (1987) and Baker (1987).

**Figure 1:**     Enteroepithelial stage in the small
                  intestine of the cat showing the luminal
                  space (L) containing cell debris, and
                  macrogametocytes (Mg) and microgametocytes
                  (M). The enterocytes also show both forms,
                  (H&E/PAS Mag X600)

**Figure 2:**     Zinc sulphate density gradient preparation
                  of Toxoplasma infected cat faeces showing
                  both sporulated (S) and unsporulated (U)
                  forms of the oocyst.
                  (Unstained Mag. X1000 Courtesy of Prof W.M.
                  Hutchison)
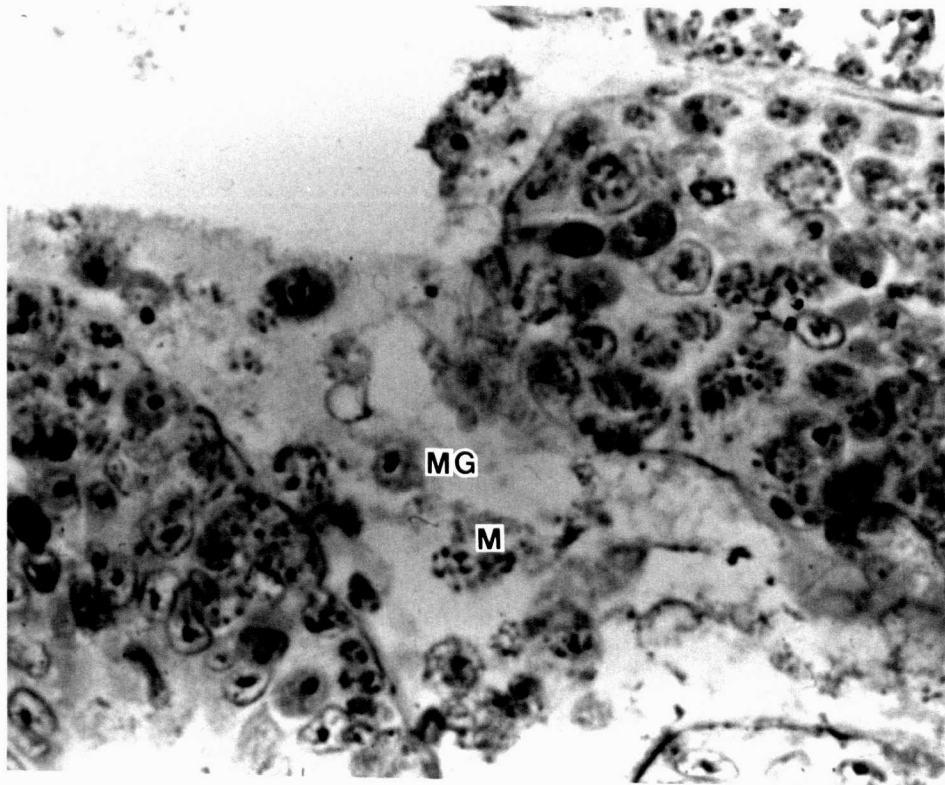
Figure 1:
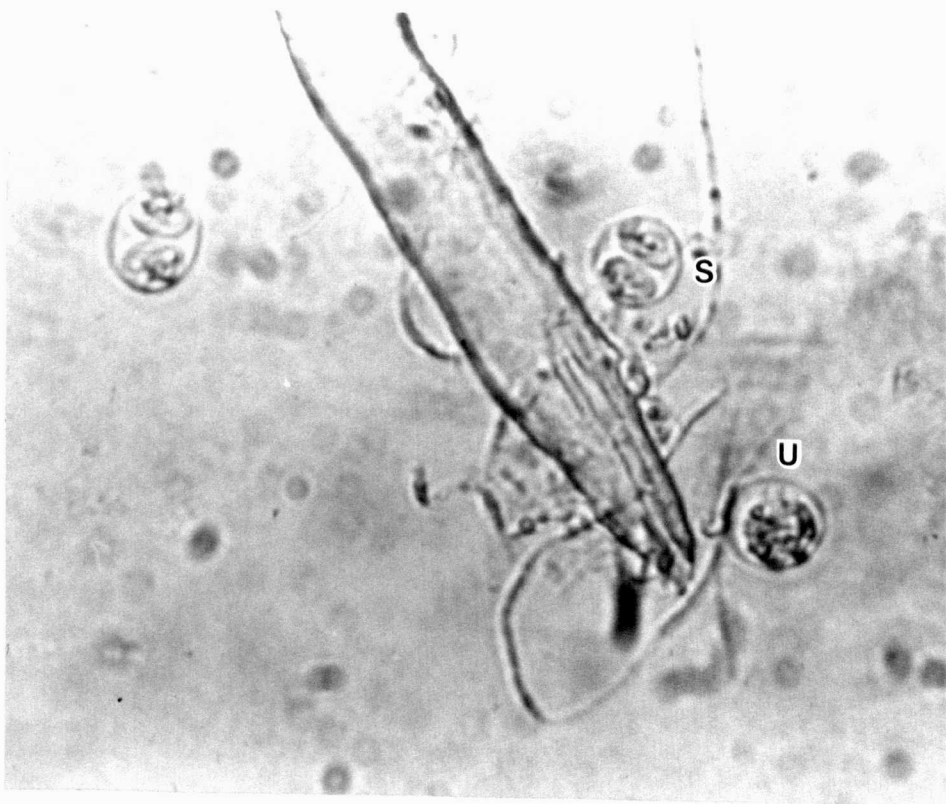


Figure 2:

**Figure 3:**     Endozoites showing characteristic "arc" shape
(PAP/Haemalum Mag. X1000)

**Figure 4:**     Mature tissue cyst in the brain of a mouse
with congenital _Toxoplasma_ infection
(Toluidine blue Mag. X 1000)

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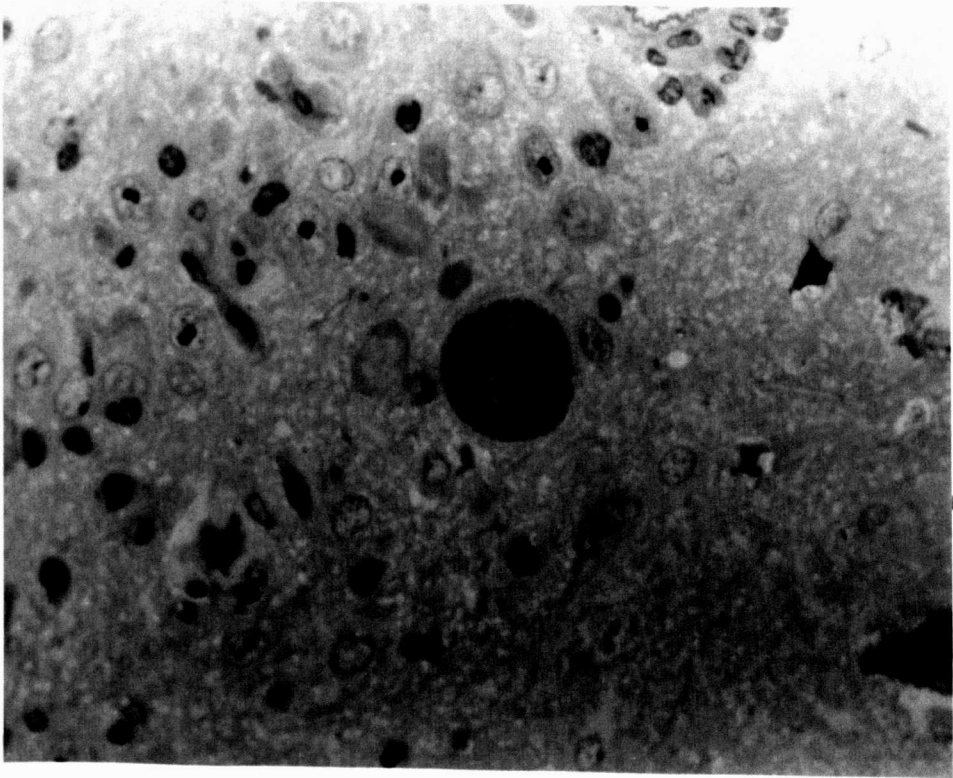


Figure 3:



Figure 4:

**Figure 5:**    Schematic representation of the modes of
dissemination of <u>Toxoplasma</u>
(After Hay 1982 and Hay and Hutchison 1983)
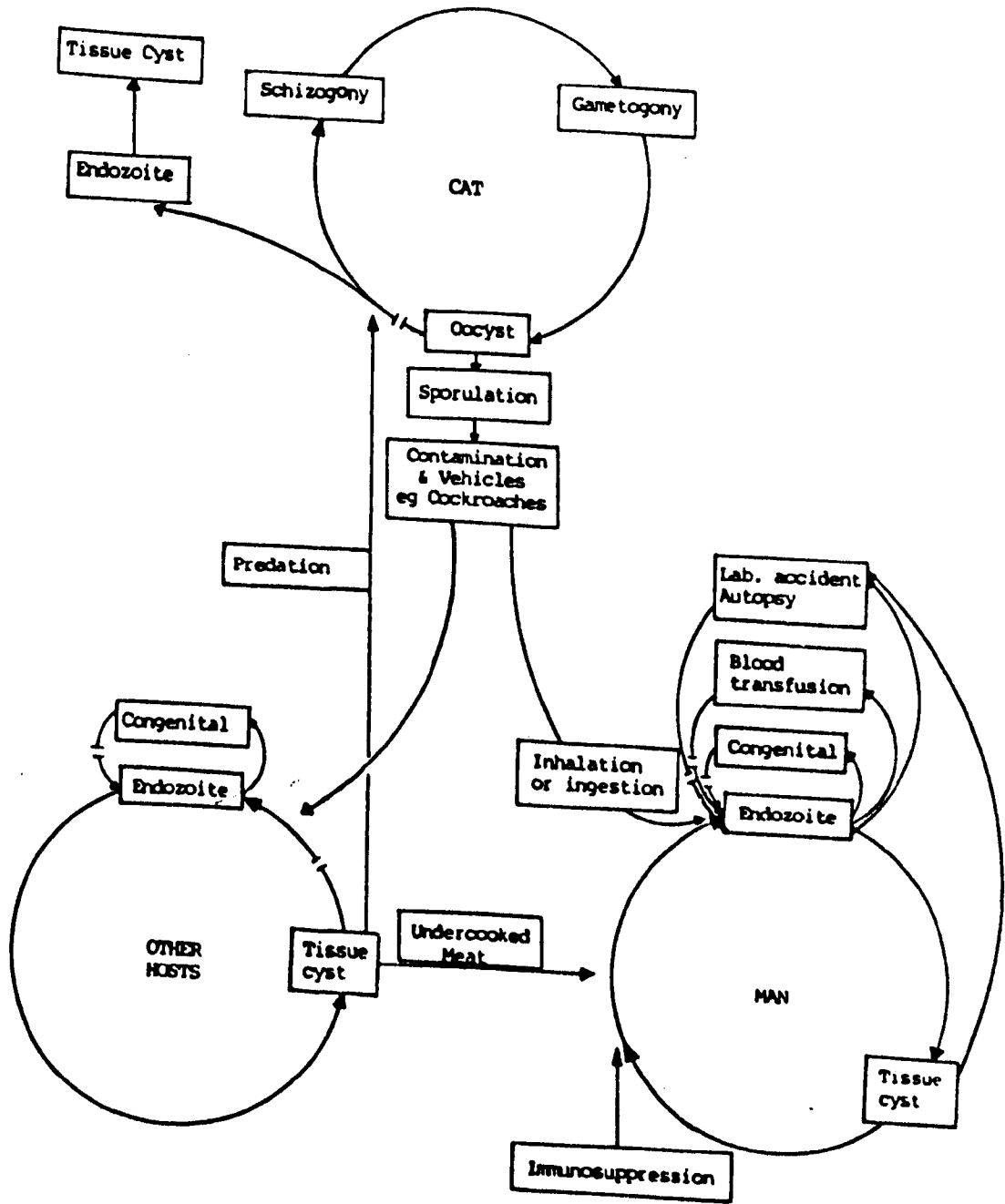
**Figure:5**

Toxoplasma exists in two distinct biotypes, both of which can occur in the cat.

First, the enteroepithelial cycle occurring in the ileum of the cat, leading to gametogeny and oocyst production with sporulation. Secondly, the extraintestinal cycle involving endozoites and tissue cyst formation occurring in extraintestinal tissues; these developmental stages constitute the whole life-cycle in mammals other than Felidae (Frenkel, 1973). A summary of the natural history and mode of dissemination of Toxoplasma is shown in Fig 5.

## 1.1.1.THE OOCYST

Schizogeny and gametogeny occur in the epithelial cells of the small intestine of the cat. Following a period of between 3 and 20 days, unsporulated oocysts are voided in cat faeces; peak production occurs between 5 and 8 days after infection (Dubey and Frenkel, 1972), with as many as 10 million oocysts being shed in a single day. Oocysts contain two sporocysts; under suitable environmental conditions oocysts sporulate in the soil within about one week (Frenkel et al 1980); they may however remain viable in moist soil for over one year. Each sporocyst contains four sporozoites; it is only this fully sporulated oocyst which is infective when ingested either by a cat, when the enteroepithelial cycle may occur, or by another warm blooded mammal, or bird, giving rise to the extraintestinal forms.

## 1.1.2. THE ENDOZOITE

Endozoites are crescentic or oval in shape with one end
pointed and the other rounded. They are usually between 4
to 8um in length and between 2 and 4um in diameter.
The nucleus is approximately central in the organism.
Endozoites are obligate intracellular organisms; they have
the potential to invade most cells. Using cinematographic
techniques, Roundanelli (1986) demonstrated that
penetration of cultured Vero cells was not the result of
passive phagocytosis; rather the process occurred by active
penetration. However, phagocytosis of the organisms by
macrophages has been observed (Jones et al. 1972).

Following cellular invasion, Toxoplasma divide rapidly
within a cytoplasmic vacuole by a process referred to as
endodyogeny (Goldman et al 1958). The cytoplasm of the
invaded cell becomes swollen and packed as a result of the
rapid division of the organism; the parasites  then
released are capable of invading adjacent cells (Lund et
al. 1961; Hirai et al. 1966; Bommer et al. 1969).

Disintegration of the invaded host cell can occur even when
the total·number of intracellular organisms is quite small
(Sabin and Olitsky, 1937); this observation suggests that
release of parasites may be dependant upon factors other
than mere multiplication of the parasite.

Endozoites can persist in a host cell for fairly long
periods without formation of tissue cysts (Remington and
Desmonts 1976). Van de Waaij (1959), suggested the term
"pseudocyst" for these structures since no host cell
components were apparently involved, or associated, with
the vacuole wall; Frenkel (1973), however, considered that
the term pseudocyst was inappropriate and that such

colonies of intracellular organisms should be defined as tissue cysts.

## 1.1.3 THE TISSUE CYST AND CYSTOZOITE

In general terms, the chronic stage of a Toxoplasma infection follows the acute stage, and is characterised by the presence of tissue cysts; these structures have been found in virtually every organ. (Remington and Desmonts, 1976). They appear, however, to be found more often in the central nervous system, retina, and skeletal and cardiac muscle Jacobs et al. (1960), It may be, however, that this observation results from the fact that these tissues have been the subject of more intense study (Remington and Krahenbuhl, 1976). Tissue cysts in the brain (Figure 4) and the retina (Figure 7) tend to be spherical in shape. In cardiac and skeletal muscle, however, they tend to conform to the shape of the muscle fibres (Figure 6, 8, 9); this probably results from movement and muscle tone.

In the early stages of development, the diameter of the tissue cyst may be of the order of 12 to 14$\mu$m; cysts may contain only 2 to 6 cystozoites (Hutchison et al. 1969). Tissue cysts may attain an approximate diameter of 100um; these cysts may contain over 3,000 cystozoites (Remington 1961; Garnham et al 1962). In mice, tissue cysts have been observed as early as eight days after experimental infection (Lainson, 1958; Hutchison et al. 1969).

Figure 6:     Skeletal muscle containing mature intact
              Toxoplasma tissue cyst. Note the oval shape
              of the cyst conforming to the shape of the
              muscle fibres
              (PAP/Haemalum Mag X 1000)

Figure 7:     Toxoplasma tissue cysts in the retina of
              a congenitally infected mouse.
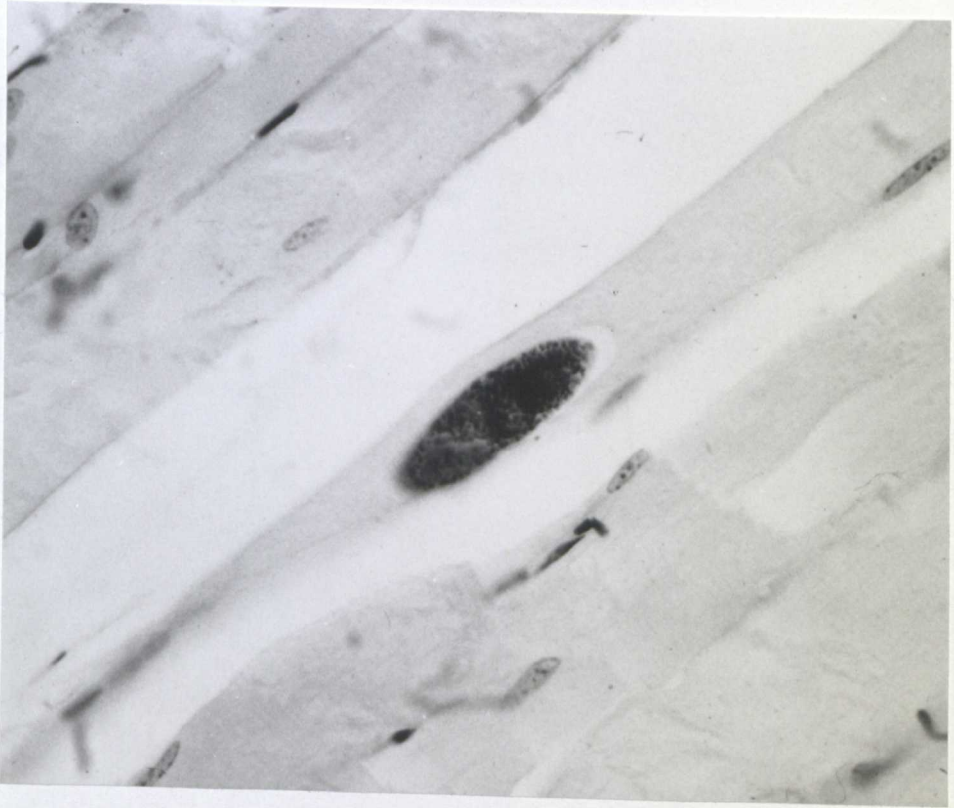              (H&E Mag. X600)

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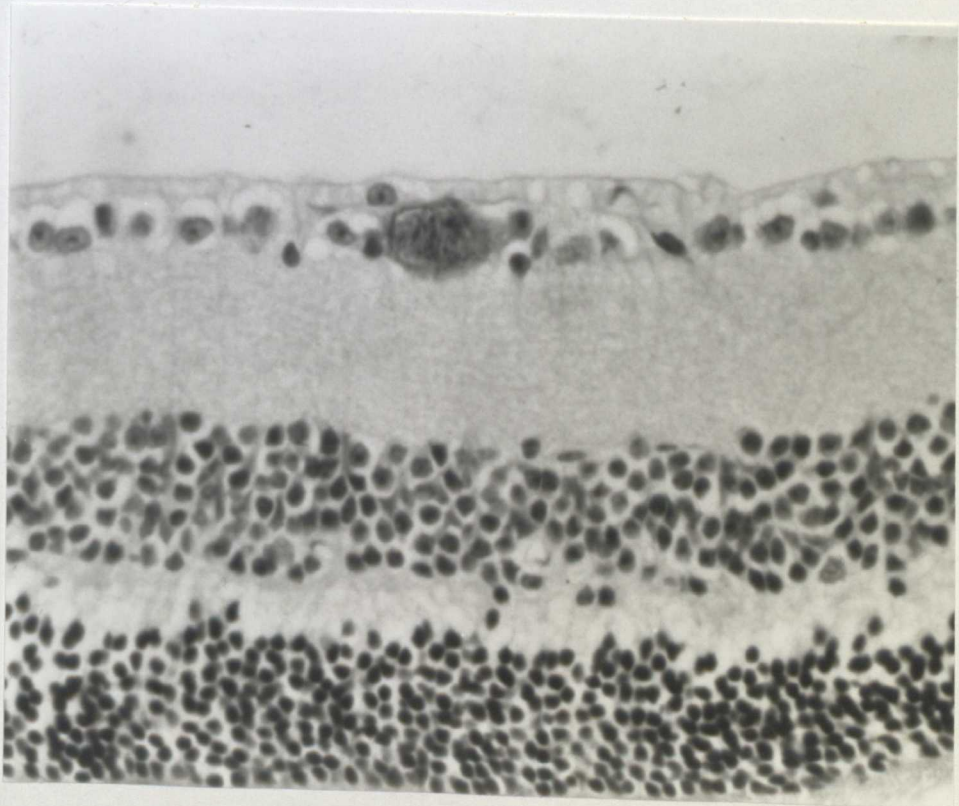


Figure 6:



Figure 7:

**Figure 8:**      <u>Toxoplasma</u> tissue cyst in cardiac muscle of a mouse infected with <u>Toxoplasma</u> in adulthood.

(PAP/Haemalum Mag. X1000)

**Figure 9:**      <u>Toxoplasma</u> tissue cyst in skeletal muscle; similar to Figure 6.

(PAP/Haemalum Mag X1000)
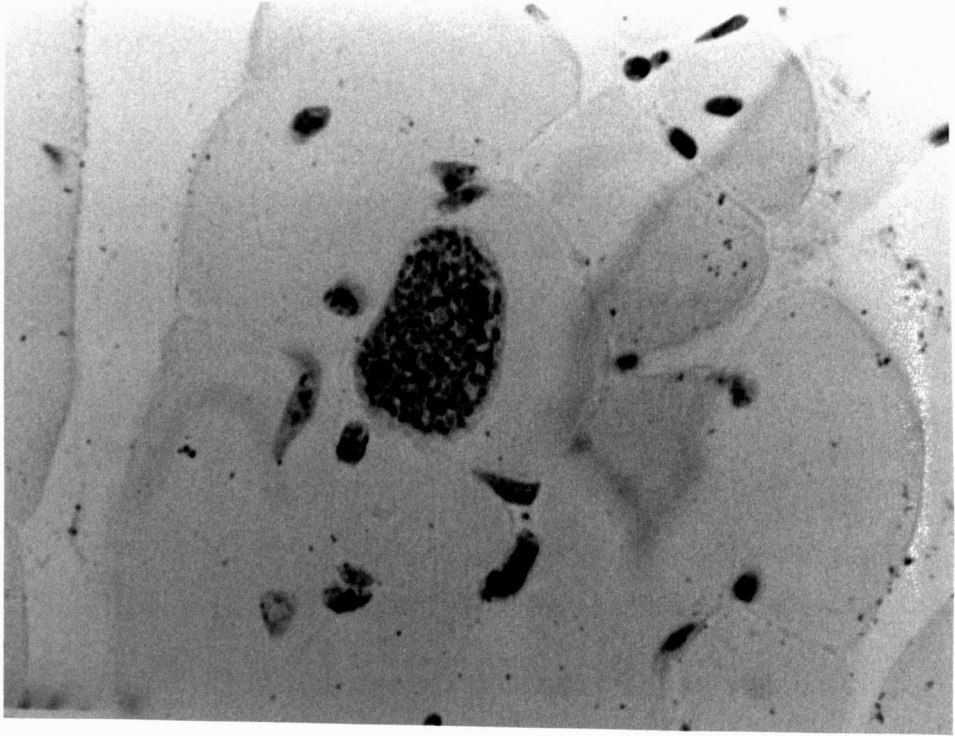
Figure 8:



Figure 9:

Cystozoites appear to multiply slowly within tissue cysts; they divide by endodyogeny (Wanko et al 1962; Zypen and Piekarski, 1966, 1967). Tissue cysts may persist as the only demonstrable form of the infection for many months; in experimental infections of guinea pigs, tissue cysts were observed after 5 years (Lainson, 1959). Thus, it is important to recognize that the presence of tissue cysts in histological sections is not an indication of a recent infection. Remington and Cavanaugh (1965) identified tissue cysts in the brains and skeletal muscles of humans with very low anti-Toxoplasma antibody titres, suggestive of a decline in antibody over time.

The precise mechanism whereby Toxoplasma can proliferate intracellularly to form tissue cysts is as yet uncertain. The development of immunity has been suggested as one contributory factor; however, tissue cyst formation in tissue culture systems, devoid of antibody or complement (Hogan et al. 1960; Jacobs, 1973), and the presence of tissue cysts in the brains of neonates (Lainson, 1958), is strongly suggestive that immunity is not the sole mechanism involved.

Shimada (1974), suggested that the formation of tissue cysts in cultured cells could be enhanced if the tissue culture medium contained anti-Toxoplasma antibodies, and especially when complement was present. These findings are consistent with those of Jira et al (1971), who showed that in mice, the number of tissue cysts increased in direct proportion to the level of antibody. Frenkel (1956) showed indirectly, that when immunity was depressed, there was renewed proliferation of endozoites; these were presumed to have originated as a result of release from tissue cysts. When immunity was restored, tissue cysts were formed. The "wall" of the tissue cyst appears to be of considerable importance for the maintenance of an environment suitable

for cystozoite proliferation; it appears to constitute a strong resistant barrier to the host immune system.

The "wall" of the tissue cyst is susceptible to disruption by the enzymes pepsin and trypsin However, the liberated cystozoites can remain viable for up to 2 hours at $4^{O}C$ in pepsin-HCl (Jacobson et al. 1960); this factor would allow the cystozoites to survive the normal digestive processes within the stomach of the host. Freezing and thawing, dessication or heating to above $56^{O}C$, the thermal death point of the organism, completely disrupts the tissue cyst. However, cystozoites can apparently survive for up to 2 months at $4^{O}C$ (Jacobs et al. 1960).

A comprehensive account of the biology of Toxoplasma is given by Dubey (1977).


## 1.2.    TOXOPLASMOSIS

Although Toxoplasma is ubiquitous in nature, the disease, toxoplasmosis, appears to be fairly uncommon.

Toxoplasmosis can conveniently be sub-divided into two broad categories: post-natally acquired infection or congenitally acquired infection.

In humans both forms of the disease are characterized by a wide variety of symptoms and clinical patterns; these can range from an almost asymptomatic course to a generalised infection with fatal outcome (Remington, 1968).

## 1.2.1.    POSTNATALLY-ACQUIRED TOXOPLASMOSIS

Infections acquired postnatally especially in adulthood appear to be the most common form of the disease (Beverley 1973). It has been estimated, serologically, that about 33% of the adult population worldwide, are or have been, infected with Toxoplasma (Kean, 1972; Frenkel, 1975). Most individuals appear to present no obvious signs or symptoms of toxoplasmosis. The most common clinical manifestations are lymphadenopathy (Siim, 1950, 1960), and fatigue without fever (Stanton and Pinkerton, 1953; Perkins, 1973). However, it must be emphasised that the infection is potentially pathogenic. It can be fatal in patients who are immunoincompetent either as a result of receiving immunosuppresive drugs for the treatment of certain malignant diseases, particularly reticuloepithelial neoplasias (Nicholson and Wolchok, 1976), and those undergoing organ transplantation (Cheever et al. 1965; Frenkel, 1971). Acquired Immune Deficiency Syndrome (AIDS) has been associated with a dramatic increase in the number of cases of toxoplasmosis. In all of these situations the disease is generally regarded as being the result of recrudescence of latent infection initially acquired postnatally (Luft and Remington, 1988; Helweg-Larsen et al. 1986). Estimates suggest that between 3% and 40% of patients with AIDS have clinically apparent toxoplasmic encephalitis. (Luft and Remington 1985). Thus Toxoplasma is becoming the most common cause of encephalitis in adults, at least in temperate areas of the world.

Comprehensive accounts of adult acquired toxoplasmosis are given by Ffrench (1965a,b) and Remington (1974).

## 1.2.2.   CONGENITAL TOXOPLASMOSIS

The first complete description of congenital toxoplasmosis in humans was given by Wolf et al. (1939).  It is generally accepted that only women who experience a primary acute infection during pregnancy can transmit Toxoplasma to their unborn foetus (Desmonts and Couvrer, 1974 a,b).  The clinical and pathological consequences of such transmission are wide ranging.  The infection may present no symptoms at birth; there may, however, be premature termination of the pregnancy or the child may be stillborn. The most commonly encountered clinical manifestations are retinochoroiditis, hydrocephalus, and intracranial calcification (Remington and Desmonts, 1983).  The stage of pregnancy at which maternal Toxoplasma infection is acquired has an important, and marked, influence on the frequency of transmission of the parasite, and consequentialy the severity of the congenital infection.  The incidence of transmission is lowest in the first trimester of pregnancy; as expected, however, clinico-pathological manifestations would be most severe at this time.  Incidence of transmission is highest in the third trimester; in this situation the congenital infection is more likely to follow a subclinical course (Desmonts and Couvreur, 1974 a,b).  A comprehensive account of toxoplasmosis in the congenitally infected infant is given by Remington and Desmonts (1983, 1990).

The precise incidence of congenital toxoplasmosis is unknown, and has been the subject of considerable debate. Beattie (1980), using serological data on pregnant women in the United Kingdom, predicted that approximately 3,300 pregnant women become infected annually; this results in about 400 cases of congenital toxoplasmosis with definite symptoms; an unknown number would present with subclinical Toxoplasma infection.

The annual cost of care and treatment of children and adults with sequelae of congenital toxoplasmosis has been estimated in 1976 to be of the order of £14 million in Great Britain (Wynn and Wynn 1976). In 1973, in the United States of America it was estimated to be of the order of $40 million (Frenkel 1973).

## 1.2.3   ANIMAL MODELS OF TOXOPLASMOSIS

Due to the nature of the disease toxoplasmosis in humans, there is an inevitable scarcity of human material suitable for the study of Toxoplasma pathogenesis. For this reason, several attempts have been made to produce suitable animal models which show similar pathology to that seen in man.

Rabbits have frequently been used in the study of ocular toxoplasmosis due to their high susceptibility and ease of ocular observation compared with smaller mammals (Beverley et al. 1955; Beverley, 1961; Nozik and O'Connor, 1970,a b). Rats were used by Kramar and Varbec, 1960, while Guinea pigs were used by Hogan et al. (1957, 1958) and Staub and Putz (1959).

Beverley (1961) described the presence of Toxoplasma tissue cysts in the retina of mice experimentally infected as adults and also in mice which were congenitally infected with the parasite. Hoffin et al (1987), produced intracranial toxoplasmosis by intracerebral inoculation of peritoneal fluid containing Toxoplasma endozoites. Ferguson and Hutchison (1988) investigated the host-parasite relationship in mouse brain following subcutanious inoculation of adult mice with intact Toxoplasma tissue cysts.

All of these animal models, however, have one major failing
in that the mode of acquisition of the infection does not
compare with that in humans. Congenital toxoplasmosis in
man is only acquired in-utero from the mother who becomes
infected with Toxoplasma for the first time during
pregnancy. Therefore, an animal model for the study of
congenital toxoplasmosis should follow the same course of
acquisition.

Hay (1979), developed such a murine model in which the
congenitally infected offspring arise from a primary
infection of the dam during gestation with the offspring
surviving the perinatal period. This model was originally
developed to study ocular toxoplasmosis (Hay et al 1981
Hutchison et al 1982, Hay 1982, Hay and Kerrigan 1982) and
behavioral changes in infected mice when compared with
uninfected controls (Hay et al. 1983 a,b,c, 1984)
Ultrastructural studies on the eyes of mice congenitally
infected in this model suggest that the pathology is
similar to that seen in man (McMenamin et al. 1986, Dutton
et al. 1986). Light microscopic studies on the brains of
these mice indicate that the changes seen are also similar
to those seen in subclinical toxoplasmosis in humans
(Graham et al. 1984). Thus the model shows every
possibility of representing true congenital toxoplasmosis
and was selected for this thesis to study the host-parasite
relationship in brain.

## 1.3. HISTOLOGICAL STUDIES CONCERNING THE HOST-PARASITE RELATIONSHIP IN TOXOPLASMA INFECTION

### 1.3.1 HISTOCHEMICAL AND TINCTORIAL METHODS

The cystozoites within the tissue cyst stain well using the periodic acid Schiff (PAS) reaction (Frenkel and Freidlander, 1951). This methodology has been used extensively for the identification of tissue cysts in histological sections.

Staining of cystozoites with the PAS reaction has been attributed to polysaccharides present within this form of the parasite. The nature of the polysaccharide is poorly defined. It has been suggested as amylopectin, possibly of parasite origin (Zypen and Piekarski, 1966; Beyer et al 1977). Meingasser et al (1977), found that it was highly acidic and diastase resistant, being associated with high levels of phosphorylase and lactate dehydrogenase. These findings together with the observation that succinic dehydrogenase and glycogen synthetase are only weakly demonstrated, led to the suggestion that the carbohydrate content was not merely an "energy store", but also acted as a biosynthetic substrate.

Beyer et al. (1977 a,b,c), performed an extensive histochemical study of cystozoites. These authors suggested that glucose liberation could be utilized in the hexose monophosphate shunt to give rise to pentoses which would contribute to the nucleic acid synthesis required for the proliferation of cystozoites.

The cyst "wall" exhibits argyrophilia and only weak PAS staining (Frenkel and Friedlander, 1951). Rodhain and Gerebtzoff (1951) used silver impregnation to study the

nature of the cyst "wall". Van der Waaij (1959), used Bodian's protargol stain for neurones, Holzer's method for glial cells, and "Azan" stains for connective tissue, in order to determine the composition of the Toxoplasma tissue cyst "wall" in mouse brain. Van der Waaij (1959) reported that most tissue cysts did not show positive staining, but occasionally intense staining was observed with Bodian's protargol stain.

## 1.3.2    IMMUNOCYTOCHEMICAL STUDIES

Immunocytochemical methods were first applied to the study of Toxoplasma by Carver and Goldman (1959). These authors used fluorescein labelled anti-Toxoplasma antiserum applied to fresh frozen, and acetic acid-ethanol fixed, Toxoplasma infected mouse brains. They demonstrated intense fluorescence associated with intact tissue cysts; they associated this phenomenon with the cyst "wall". The latter authors also described considerable fluorescence in the host brain adjacent to the intact tissue cyst and suggested that this was probably due to antigen "leakage". Furthermore they suggested that since there was intense staining of the cyst "wall", this structure must be of parasite origin. However, carrying out the same procedure using formalin-fixed paraffin-embedded sections provided no immunostaining of the host tissues; this was suggested to indicate that Toxoplasma antigen was fixative labile.

Huldt (1971) also used immunofluorescence to study peritoneal exudates from different strains of Toxoplasma in frozen sections of Guinea pig tissue. Antigen "leakage" from intact tissue cysts was again observed; the authors suggested that this was a potential mechanism to explain the maintenance of a high anti-Toxoplasma antibody titre in

the chronic stage of infection, that is in association with intact Toxoplasma tissue cysts.

Rao and Font (1977) demonstrated Toxoplasma tissue cysts in the eye using immunofluorescence. The latter study was based on the tissue derived from an 82 year old man who presented with bilateral uveitis requiring enucleation as a result of blindness and pain. Considerable variation in staining intensity was observed. This led to the conclusion that there were differences in tissue cyst viability; so called "necrotic" cysts were detected using electron microscopy; "free" organisms were also seen within the retina.

Conley and Jenkins (1981) described a peroxidase-antiperoxidase (Sternberger et al. 1970), immunocytochemical study of the anatomical relationship of Toxoplasma antigen to the inflammatory response in the brains of mice with chronic Toxoplasma infection. When the tissue cysts were intact, clear, intense staining of the tissue cyst "wall" and contained cystozoites was observed. Staining of cystozoites was greatest on their surface. This was seen especially in tissue cysts where the cystozoites were most tightly packed. In degenerate tissue cysts and in necrotic foci, however, the detection of antigen was not so clear; staining appeared to be irregular and variable. It was not possible to positively identify endozoites by shape or form, but large quantities of irregular antigenic material was seen. Conley et al (1981) used Sternberger's exquisitely sensitive peroxidase-antiperoxidase method, to study Toxoplasma infection in paraffin-processed brain tissue from humans with toxoplasmic encephalitis. These workers were able to demonstrate intense staining of the intact tissue cyst "wall" and of cystozoites; the staining of the cystozoites was most intense on their surface. Endozoites, however,

showed only patchy staining with ill-defined remnants; irregular antigen staining was also observed within inflammatory foci. Kittas et al. (1984) used the peroxidase-antiperoxidase method, with anti-Toxoplasma as the primary layer, to study the changes induced in the brains of mice chronically infected with Toxoplasma. The PAP technique was able to detect tissue cysts at a much earlier stage of development than conventional light microscopy or immunofluorescence. Kittas et al. (1984) were able to detect more tissue cysts by this method. Furthermore, the number of tissue cysts increased in direct proportion to the duration of infection.

Hay et al (1985) used the peroxidase-antiperoxidase method to study the distribution of Toxoplasma antigen in the eyes of mice with congenital ocular toxoplasmosis. In this study only cystozoites were demonstrated. No staining of endozoites or extracystic antigen was discovered (Graham et al. 1984), extended earlier work on the eye in their model of congenital toxoplasmosis (Hay et al. 1981) to a study of the brain. Only cystozoites showed intense staining with the PAP. Weak staining was described in the cyst "wall" but, as with the eye no extracystic antigen was detected.

Bjerkas and Landsverk (1986) compared the peroxidase-antiperoxidase method with the avidin-biotin complex (ABC) method (Guesden et al. 1979; Hsu et al. 1981; Childs and Unabia, 1982), as a means of detecting Toxoplasma antigen in archived formalin-fixed paraffin-wax embedded tissue. These authors concluded that the PAP was equally effective, if not more effective than the ABC method. Furthermore, they performed electron microscopy on the paraffin processed samples which gave positive immunocytochemical staining. Due to the inappropriate fixation, however, the quality of ultrastructural morphology was poor, although the authors were confident that Toxoplasma was identified.

Lunde and Jacobs (1983) prepared fluorescein-labelled anti-endozoite and anti-cystozoite antisera and applied them to preparations containing either Toxoplasma endozoites or cystozoites. They demonstrated that the anti-endozoite antiserum reacted best with endozoites, but only weakly with cystozoites. However, the anti-cystozoite antiserum did not react with endozoites, but showed intense staining of the cystozoites. These authors concluded from these findings that there was a distinct antigenic variation between endozoites and cystozoites of the same strain of Toxoplasma. In a subsequent study Lunde and Jacobs (1985) used the same methodology to demonstrate endozoite and cystozoite antigens in tissue culture preparations. As a control in this experiment, anti-endozoite antiserum was absorbed using endozoites. This resulted in negative staining of endozoites; cystozoites, however, remained only weakly stained, thus confirming the previous findings. Lunde and Jacobs (1985) demonstrated that cystozoites lost their surface staining after only a few generations in-vitro.

### 1.3.3. ULTRASTRUCTURAL STUDIES

Gustafson et al. (1954), observed the ultrastructural development of the tissue cyst. They concluded that during the early stages of development of the tissue cyst, filamentous and granular material became associated with the primary cyst "wall" leading to increased argyrophilia. Garnham et al (1962), speculated on the origin of the Toxoplasma tissue cyst "wall".They suggested that it originated from the organism, and not from the host. This study was based on the use of resin embedded impression smears of infected mouse brain. Despite the preparatory technique, the specimen preservation was of good quality, a feature which resulted in the first description of

invagination of the primary cyst "wall". Wanko et al. (1962), in a study of Toxoplasma infected mouse brains, suggested that the tissue cyst "wall" was composed of elements derived from microglial cells as well as rough endoplasmic reticulum derived from these cells. They observed separation of the larger tissue cysts from the host brain tissue making it difficult to positively identify the host cell type. This separation was interpreted as a preparation artefact. Despite this artefact Wanko et al.(1962) stated that the host cell was the astrocyte.

Gavin et al. (1962) used electron microscopy to study the reproduction of cystozoites. They confirmed the findings of Gustafson et al. (1962) in that the origin of the tissue cyst "wall" appeared to be the pellicle. Matsubayashi and Akao (1963) using in-vitro techniques, gave an excellent description of the ultrastructure of the cystozoite. They suggested that the tissue cyst "wall" was of parasite origin being derived from the aggregation of the granular material found between the cystozoite, and probably secreted by them. They also described the occurrence of large spaces between cystozoites in some cysts; this was suggestive of packing of cystozoites, and was used as an indication of cyst maturity. The outermost component of the tissue cyst wall in the Matsubayashi and Akao (1963) study, was composed of mitochondria, endoplasmic reticulum and fine granular material.

Van der Zypen and Piekarski, (1966) gave an account of the ultrastructure of intact tissue cysts in-vitro. They described the parasitophorous vacuole membrane as an invaginated membrane with numerous infolds. They also observed that as the tissue cyst matured the cytozoites became more tightly packed. They concluded that the

breakdown of the tissue cyst was a result of decreasing elasticity of the host cell.

Calloway et al. (1968) performed an ultrastructural study of brain tissue from a renal transplant patient who died of toxoplasmic encephalitis. Using both fixed, and fresh-frozen brain material, considerable packing of cystozoites was observed in what appeared to be mature cysts. No reference was made of the possible host cell component. The large numbers of tissue cysts observed in most organs was attributed to immunosuppression in the patient. Bommer et al. (1969), using time-lapse cinematography on in-vitro cultures of Toxoplasma infected cells in order to investigate the dynamics of parasite invasion, observed tissue cyst formation and eventual disruption of tissue cysts. The study was supported by electron microscopic examination of infected cells at different stages of development. It was concluded that initially there was a passive leakage of individual cystozoites and parasitic material from infected cells; at a later stage in development, cell breakdown occurred, presumably as a result of a loss of elasticity in the host cell. In another cinematographic and electron microscopic study, Roundanelli et al. (1986) obtained similar results; they further suggested that the initial penetration of the host cell by the endozoites was an active process, not merely passive phagocytosis.

Results obtained from an ultrastructural study of brain tissue from three cases of human adult acquired toxoplasmosis led Ghatak et al. (1973) to dispute the occurrence of a host cell component in intact tissue cyst. However, in a subsequent study (Ghatak and Zimmerman 1973), a host cell component said to be of astrocytic origin, was described. This alteration in interpretation was based entirely on light microscopic observations, since the

ultrastructural preservation was extremely poor.
Furthermore, no specific staining method for astrocytes was
employed by Ghatak and his co-workers.

Hammond (1973) presented a review of the ultrastructure of
coccidia. In this work it was suggested that the
invagination of the parasitophorous vacuole membrane in
early development of cysts in the tissue cyst forming
coccidia occurred in order to accommodate absorption of
nutrients from the host, which would be required by
cystozoites during their development.

Scholtyseck et al. (1974) compared the ultrastructural
morphology of Sarcocystis and Toxoplasma tissue cysts in
skeletal muscle. They stated that Toxoplasma tissue cysts
showed variation with age. In younger cysts the
parasitophorous vacuole membrane was formed from the host
cell; in older, more mature cysts, the cystozoites
themselves were surrounded by an amorphous ground matrix
which aggregated together to from the parasitophorous
vacuole membrane.

In a review of host cell invasion by coccidia, Long and
Speer (1977), concluded that the breakdown of tissue cysts
occurred as a consequence of loss of elasticity in the wall
of the cyst. Prior to this breakdown, there would be
passive release of parasites through the intact cyst
"wall". However, no explanation as to how this might be
achieved was presented. The principal result used to
arrive at these conclusions were those of Bommer et al.
(1969), cited above.

Melhorn and Frenkel, (1980) performed an ultrastructural
comparison of Toxoplasma, Sarcocystis muris and Hammondia
in skeletal muscle. They reported that there was no
obvious morphological difference between tissue cysts of
Hammondia and Toxoplasma; the cystozoites of each organism

were also indistinguishable. There was, however, variation in the degree of convolution of the parasitophorous vacuole membrane with age.

Uga et al. (1980), examined ultrastructurally, tissue from the brains of mice that had been infected by intracranial inoculation with brain homogenates from other Toxoplasma infected mice. They were able to detect, and examine, brain cysts within days of inoculation. The tissue cyst was said to be composed of host cell components derived from destroyed parasitized cells. Packing of cystozoites within tissue cysts occurred very early in the infection. It was concluded that this occurrence should not be regarded as a sign of cyst maturity. This conclusion was contrary to that of Matsubayashi and Akao (1963), cited above. Antibody levels were measured throughout the life of the mice; as antibody levels increased, so the number of observed cysts increased. This might suggest that tissue cyst formation is brought about as a response to increasing antibody levels.

Shimizu et al. (1985) studied Toxoplasma tissue cysts in mouse retina. They described two distinct types of tissue cyst. One had no apparent inflammatory response within its immediate vicinity; the other was associated with considerable inflammatory response surrounding the tissue cysts, but with no obvious breakdown of the tissue cyst in association with this infiltrate. Both types of tissue cyst were seen in all layers of the retina. Dutton et al. (1986) and McMenamin et al. (1986) investigated ultrastructurally the retina of a murine model of congenital toxoplasmosis (Hay et al. 1981). Unlike Schimizu et al. (1985) these authors did not detect any inflammatory infiltrate associated with tissue cysts. In one instance the host cell component in the retina was thought to be a Muller cell; these cells are modified astrocytes. However, most cysts were observed within cells which had the cytoplasmic morphology of the neurone.

An ultrastructural study of the host-parasite relationship in adult mouse brain was performed by Ferguson and Hutchison (1987a,b) on mice which were infected by peritoneal inoculation with a homogenised brain extract containing Toxoplasma tissue cysts. In the latter study 50 cysts were examined: 90% were seen in grey matter; 10% were detected in white matter.


## 1.3.4.    ELECTRON IMMUNOCYTOCHEMISTRY

Several workers have used electron immunocytochemistry in order to investigate the distribution of Toxoplasma antigen within intact tissue cysts. Matsubyashi and Akao (1966) used ferritin-conjugated anti-Toxoplasma IgG to detect surface antigens on endozoites. When isolated intact tissue cysts were examined, these authors were unable to detect antigen within the tissue cysts. They concluded that antibodies were not able to penetrate intact isolated tissue cysts. When the cystozoites were sectioned, however, an increased level of antigen in the vicinity of the tissue cyst "wall" was observed. This lack of identity of antigenic material was proposed as a possible explanation for the absence of an inflammatory response in vivo.

Using ferritin-labelled specific IgG and IgM to locate the antigenic sites on the surface of Toxoplasma taken from the peritoneal exudate of infected mice, Carosi et al. (1980) showed that deposition of labelled anti-IgG occurred only on the endozoite surface and in tissue cysts where there was obvious disruption of the host cell membranes. Tissue cysts which did not show such disruption, failed to show any deposition of either IgG or IgM. These findings were in agreement with those of Matsubyashi and Akao (1966),

cited above. Johnson et al. (1983) used peroxidase-
labelled monoclonal antibodies to seven Toxoplasma surface
antigens and demonstrated that in RH strain endozoite at
least, surface antigens were exclusively protein in nature,
were of cytoplasmic origin and were secreted by the
parasite onto their surface.

## 1.4    AIMS OF THE PRESENT STUDY

There remains considerable confusion and contradiction regarding the nature of the host-component of the intact tissue cyst "wall" and the inter-relationship between _Toxoplasma_ antigen and the host tissue in the vicinity of the intact tissue cyst. A number of previous observations are complicated by poor cellular preservation. _In vitro_ studies, undertaken in an attempt to overcome this problem, cannot be regarded as fully representative of the _in vivo_ situation. This is especially so with the congenital form of _Toxoplasma_ infection where there is an inevitable scarcity of human material for histological examination. Thus, the primary purpose of this present study was to examine, principally at the ultrastructural level, the nature of the interface between cystozoites within intact tissue cysts and the brain of a murine model of congenital toxoplasmosis Hay _et al_. (1981) It was also proposed that on morphological grounds, it may be possible to relate inflammatory changes in the brains of such mice to subsequent breakdown of the tissue cyst.

CHAPTER 2

MATERIALS AND METHODS

## 2.1    MICE

Albino STR strain (Hutchison 1986) mice were used
throughout this work.  The mice were descendents of a
breeding nucleus obtained by the University of Strathclyde
Pharmacy Department from the Imperial Cancer Research Fund
London in 1954.  A population of these mice has been kept
in the Leicester Polytechnic Department of Pharmacy since
1984.  No naturally occurring infection with Toxoplasma has
ever been found either serologically or parasitologically
in several thousand of this mouse strain which have been
examined (Hay, personal communication 1989).

## 2.2. STRAIN OF TOXOPLASMA

In all experiments, a Toxoplasma strain of low virulence
was used; the Beverley or RRA strain (Beverley 1959).
Morbidity is rarely caused by this strain of Toxoplasma in
inoculated mice.  However, ill health is occasionally
exhibited, during the second and third weeks post-infection
in adult mice (Hay, 1982).

## 2.3. MAINTENANCE OF MICE

A maximum of six mice of the same sex were housed in Type
INKP Cages (high density polypropylene boxes 33 x 15 x 13
cm, with stainless steel wire lids).  Constant conditions
of temperature ($21\pm2^{\circ}C$) and humidity ($50\pm10\%$ RH) were
maintained throughout the experiments.

The mice were fed with Labsure animal Diet CRN nuts.
(Rank-Hovis-MacDougall) which contained recommended levels
of Vitamin A and E.  Water was provided ad libitum from
drop bottles.  A 12-hour dark-light cycle was maintained
throughout the experiments.


## 2.4.      PREPARATION OF BRAIN INOCULUM

The method used was similar to that described by Hay (1979,
1982).  Emulsified brain tissue from mice infected 17 weeks
previously was appropriately diluted with isotonic saline
to give a standard inoculum of 10 cysts per 0.2ml.

### Method

1.    The brain of a mouse that was infected with the RRA
      strain of Toxoplasma 17 weeks previously, was removed.

2.    The brain was homogenised in a sterile mortar with
      a small quality of sterile isotonic saline (0.9%) and
      aspirated into a sterile 1ml hypodermic syringe.

3.    One drop of the homogenate was transferred onto a
      microscope slide and a coverslip applied.

4.    The number of cysts (Y) was counted in the complete
      area beneath the coverslip (ie. the total number of
      cysts in the drop).

5.    The total number of drops (X) in the syringe was
      counted.

6.  The total volume (V) of the homogenate in the syringe was determined.

## Calculations.

Where X = number of homogenate drops
       Y = number of cysts per drop

The number of cysts in the brain = Y.X.
To produce 10 cysts per 0.2 ml inoculum, make the volume Vml up to Y.X/50ml.

## 2.5.    PREPARATION OF CONGENITALLY INFECTED MICE

The procedure for the preparation of mice for this investigation was carried out as described by Hay (1979,1982).Virgin female mice were mated at approximately seven weeks of age. Copulation was determined by the presence of a vaginal plug. Twelve days after the observation of the plug, each experimental mouse was subcutaneously injected with 10, 17-week old Toxoplasma tissue cysts. Each control mouse was injected with 0.2ml of non-infected mouse brain extract in saline.  Pregnant females were observed closely towards the end of gestation. Offspring were separated from their respective dams immediately after birth and fostered to uninfected lactating dams.  This procedure prevented the offspring becoming infected via the lactating dam's milk; the latter is known to transmit Toxoplasma to the suckling young (Eichenwald, 1948; Remington et al. 1961a; Rommel and Breunig, 1967).  The presence of infection in the offspring was determined using the direct agglutination test (Desmont and Remington, 1980) for anti-Toxoplasma antibodies (Section 2.7)

## 2.6     PREPARATION OF BRAIN TISSUE FOR HISTOLOGICAL EXAMINATION

Mice prepared as described above and aged between 14 and 20 weeks were anaesthetized by carbon dioxide inhalation. A thoracotomy was performed, and a cannula inserted into the proximal aorta via the left ventricle. The right atrium was then incised and blood collected from the thoracic cavity. This was used for serological examination for anti-Toxoplasma antibodies. The mice were fixed by whole body perfusion. This was performed by initially perfusing the animal with warm (37°C) heparinized saline, followed by either warm 10% phosphate buffered formalin or Karnovsky's glutaraldehyde-formaldehyde fixative (1965). The dissection procedure was based on that described by Graham et al. (1984). The head of each mouse was then separated from its body by an incision passing approximately 5mm posterior to the occipital bone. The brain was then separated from the spinal cord at the level of the foramen magnum. The top of the skull was carefully removed and the brain lifted up gently to allow severing to the optic nerves at the level of the optic chiasma. The brain was removed and placed into either 10% buffered formalin or Karnovsky's fixative. Brains for immunocytochemistry studies, which were to be performed on fresh frozen cryostat sections, were removed without prior whole body perfusion with fixative. Slices of unfixed brain were placed onto cork discs using OCT cryoprotector (Miles Laboratories Ltd.) and plunged into isopentane cooled to -150°C using liquid nitrogen. The frozen blocks of tissue were then stored in liquid nitrogen until required.

## 2.7. DIRECT AGGLUTINATION TEST

The direct agglutination test was performed in disposable polystyrene microtitre plates with 96 U-shaped wells (Biomerieux).

### Agglutination antigen

Stock antigen comprised formalin-killed endozoites at a concentration of $12 \times 10^6$ organisms per ml. This stock suspension was further diluted 1/12 with bovine albumen borate saline (BABS) buffer pH 8.9, giving a working concentration of $1 \times 10^6$ organisms per ml.

### Bovine Albumen Borate Saline Buffer pH 8.9

Sodium chloride        7.012 g.
Boric acid             3.092 g.
Sodium hydroxide (M)   24 ml.
Bovine albumen         4.0 g
Sodium azide           1.0 g
Make up to one litre with distilled water and store at 4°C.

### Method

1. 20ul. of whole blood or serum was added to 150ml of phosphate buffered saline pH 7.2, in the stock well.
2. The plate was shaken and left to allow the cells to settle; the supernatant was considered as the original serum dilution (approximately 1/15).
3. Three wells each received 50µl of 2-mercaptoethanol (BDH) diluted to 0.2M in PBS.
4. 10ul of the original serum dilution was transferred to the second well in the series to obtain a dilution of 1/6.
5. This dilution was serially diluted into the other two wells to produce dilutions of 1/36 and 1/216. 50ul of the

agglutination antigen was added to each well, the plates
were shaken and allowed to settle overnight before being
read the following day.

## Results

Agglutination was shown by a diffuse layer of parasites or
an irregular shaped button. A clearly defined button
indicated a negative result. The highest dilution of the
serum which produced disruption of the button with an
irregular periphery was considered to be the endpoint.

## 2.8 .    LIGHT MICROSCOPY

Mice were anaesthetised prior to whole body perfusion with
formal-acetic methanol fixative (Graham et al 1984).
Brains were removed and processed into paraffin
wax using the protocol described in section 2.10. Sections
were cut at approximately 4um and stained with Haematoxylin
and Eosin (H&E).  Sections containing intact tissue cysts
were stained with the periodic acid Schiff method (PAS),
the methenamine silver method (Gomori, 1946), Palmgren's
argyrophilia method (Palmgren, 1948), and the protargol
silver method (Bodian 1936) as modified by Clarke (1973).
Protocols for these staining methods are given in section
2.11.

## 2.9.    ELECTRON MICROSCOPY

Anaesthetised mice were whole body perfused with
Karnovsky's formaldehyde-gluteraldehyde fixative,
(Karnovsky, 1965).  Brains were removed and immersed in
fresh Karnovsky's fixative for 2 hours at 4$^{o}$C.  Selected
blocks of less than 1mm$^3$ were post-fixed in 1% osmium

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tetroxide, immersed in 1% uranyl acetate and embedded in epoxy resin, following the protocol described in section 2.12. Semi-thin sections (0.5um) were cut on an ultramicrotome using glass knives and stained with 1% toluidine blue in 1% aqueous borax. Ultra thin sections (50-90nm) containing intact tissue cysts were mounted onto 200 mesh copper grids, contrasted with lead citrate and examined using a JEOL 100CX Temscan electron microscope.

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## 2.10.    PARAFFIN PROCESSING SCHEDULE

All tissue for paraffin wax embedding were processed on an automatic tissue processor (Shandon Histokinette). using the following schedule:


1. Tissues were placed into 50% aqueous isopropanol (IPA) for two hours.
2. Tissues were transferred to 70% IPA for two hours.
3. Tissues were transferred to 90% IPA followed by 95% IPA, one hour in each.
4. The tissues were given three changes of absolute IPA one hour each and then transferred into xylene for one hour, followed by fresh xylene for a further three hours.
5. Tissues were transfered into molten paraffin wax, melting point 56°C (BDH), for three hours and then transferred into fresh molten paraffin wax for a further three hours.
6. Fully infiltrated tissue blocks were embedded in fresh molten paraffin wax and cast into TISSUE TEK embedding moulds (Miles Laboratories)

## 2.11    LIGHT MICROSCOPE STAINING METHODS

### 2.11.1.    <u>MAYERS HAEMATOXYLIN AND EOSIN</u>

1.Sections were dewaxed in xylene for one minute and transferred to distilled water via absolute isopropanol (IPA) for one minute.

2. The sections were stained in Mayer's haemalum for four to six minutes.

3. Following a quick rinse in distilled water the nuclei were "blued" using Scott's tap water substitute (BDH), for one minute.

4. Sections were rinsed in distilled water, and then stained in 1% aqueous eosin (BDH) for three minutes.

5. Following a brief wash in tap water, the sections were dehydrated in IPA and cleared in xylene prior to mounting in XAM resinous mounting medium (BDH)

RESULTS    Nuclei- blue

Cytoplasm and connective tissue shades of pink.

### 2.11.2.    <u>PERIODIC ACID SCHIFF METHOD</u>

1. Sections were brought to distilled water as described in section 2.11.1., then transferred into 1% aqueous periodic acid (BDH) for five minutes.

2. Following a wash in running tap water, the sections were rinsed in distilled water, then treated with Schiff's reagent (BDH) for ten to twenty minutes.

3. Sections were washed in running tap water for at least twenty minutes and then counterstained with Mayer's haemalum for three minutes.

4. The nuclei were "blued" as described in section 2.11.1 then dehydrated in IPA, cleared in xylene and mounted in XAM resinous mountant (BDH)

RESULTS:    Polysaccharides and neutral mucins- magenta
            Nuclei- Blue.


## 2.11.3.   GOMORI'S HEXAMINE SILVER METHOD

### Stock Hexamine silver solution.

Five ml of 5% aqueous silver nitrate (Analar BDH) and 100ml of 3% aqueous hexamine (Analar BDH),were mixed together. A white precipitate formed which dissolved on mixing. This solution was kept in a dark stoppered bottle at 4°C for up to 2 months.

### Working solution.

Two ml of 5% aqueous sodium tetraborate (Analar BDH) was diluted with 25 ml of distilled water. 25ml of the stock hexamine-silver solution was added and the reagent used immediately.

### Method

1. Sections were taken to distilled water as described in section 2.11.1. and treated with 1% aqueous periodic acid (Analar BDH), for five minutes prior to washing well in distilled water. The sections were then placed in pre-heated hexamine silver solution in a 56°C water bath, examined after twenty minutes, and subsequently until the basement membrane and carbohydrates were blackened (approximately twenty to forty minutes).

2. Following several changes of distilled water, the sections were toned in 1% aqueous yellow gold chloride (Analar BDH) for two to five minutes.

3. The sections were washed well in distilled water, then fixed in 5% aqueous sodium thiosulphate (Analar BDH) for five minutes.

3. Sections were counterstained with 1% aqueous light green in 0.2% acetic acid for one minute, washed in distilled water, dehydrated in IPA, cleared in xylene and mounted in XAM resinous mountant (BDH)

RESULTS:   Polysaccharides and neutral mucins-Black
           Nuclei and connective tissue- Green.


## 2.11.4.   PALMGRENS SILVER METHOD

### Solutions required.

### Acid Formalin

25% aqueous formalin with 0.2ml 1% aqueous nitric acid.

### Silver solution

15% silver nitrate (Analar BDH) in 10% aqueous potassium nitrate (Analar BDH) with 1ml 5% aqueous glycine added.

### Reducer

10% pyrogallol (BDH) in 55% aqueous ethanol with 2ml 1% nitric acid.

### Toner

0.5% aqueous yellow gold chloride (Analar BDH) with 0.1% aqueous glacial acetic acid.

## Intensifier

Add 2 drops of analine oil to 100ml of 50% aqueous ethanol.

## Method

1. Sections were dewaxed as described in section 2.11.1. coated with a layer of 1% nitrocellulose and taken to distilled water.

2. Sections were treated with the acid-formalin solution for five minutes, then washed well with three changes of distilled water and placed in the silver solution for fifteen minutes at room temperature.

3. Without rinsing, the slides were drained and placed into the reducer solution, which had been pre-heated to $40^{o}C$ for one minute and agitated vigorously. For this procedure one Coplin jar was used for each slide.

4. The slides were transferred to the intensifier, then rinsed in several changes of distilled water.

5. The sections were examined microscopically; if necessary stages 2 to 4 were repeated.

6. The sections were toned in gold chloride for five minutes then transferred to fresh intensifier for fifteen seconds with vigorous agitation.

7. Following microscopic examination, stage 5 was repeated if necessary.

8. The sections were fixed in 5% sodium thiosulphate for fifteen seconds, rinsed in distilled water, dehydrated in IPA, cleared in xylene and mounted in XAM resinous mountant (BDH).

**Results**   **Neurones-BLACK**
**Background-BROWN**

## 2.11.5.   BODIAN'S COPPER-PROTARGOL METHOD

### Solutions required.

### Copper-protargol solution.

Two grams of clean copper foil (BDH) cut into 2cm pieces were added to 50ml of 1% aqueous Protargol (Roques)

### Primary reducer

1% aqueous hydroquinone in 5% sodium sulphite (Analar BDH) was prepared fresh, immediately before use.

### METHOD

1. Sections were taken to distilled water as described in section 2.11.1. and incubated overnight at 37°C in protargol solution in a clean Coplin jar.
2. Sections were washed several times in distilled water, then placed in the primary reducer for ten minutes.
3. The sections were washed well in distilled water, then running tap water for at least ten minutes, followed by a final rinse in distilled water.
4. The sections were treated with 1% aqueous yellow gold chloride (Analar BDH), then washed well in distilled water.
5. Following treatment with 2% aqueous oxalic acid (Analar BDH), the sections were fixed in 5% aqueous sodium thiosulphate (Analar BDH), washed in water, dehydrated, cleared and mounted in XAM resinous mountant (BDH).

### Results   Neurones-BLACK
            Background-BROWN

## 2.12. ELECTRON MICROSCOPIC PROCESSING SCHEDULE FOR EPOXY RESIN.

1. Tissue was diced into 1mm cubes on dental wax using a sharp new razor blade, and given two washes in sodium cacodylate buffer (pH 7.2), ten minutes in each.

2. Tissue was fixed in 1% osmium tetroxide for 1 hour at $4^{o}C$, and washed in two changes of sodium cacodylate buffer, ten minutes in each.

3. Tissue was dehydrated with a graded series of ethanol, commencing with 70%, followed by 90% and 95%, each for ten minutes.

4. The tissue was transferred to three changes of absolute ethanol, ten minutes each, and placed into a 50/50 mixture of ethanol and propylene oxide (BDH) for ten minutes.

5. This was replaced by two changes of fresh propylene oxide for ten minutes each followed by a 50/50 mixture of propylene oxide and EMIX epoxy resin (Polaron) at $37^{o}C$, for one hour.

6. Tissue was transferred to neat epoxy resin at $37^{o}C$ for one hour, then cast into the final resin solution and polymerized at $56^{o}C$ overnight.

## 2.13. IMMUNOCYTOCHEMISTRY LIGHT MICROSCOPY

For immunocytochemical studies, brains were removed and post-fixed in 10% formalin containing 5% glacial acetic acid, dissected as described in section 2.6., then embedded in paraffin wax as described in section 2.10.

## 2.13.1    PEROXIDASE-ANTI-PEROXIDASE PROTOCOL

The protocol for the peroxidase anti-peroxidase (PAP) method was as follows:

1    Sections (4um thick) were dewaxed with xylene and rehydrated through graded IPA.

2    Endogenous peroxidase activity was blocked by immersing sections for 20 minutes in a solution of 0.5% hydrogen peroxide in methanol.

3    Sections were washed in two changes, 10 minutes each, of Tris buffered saline (TBS, pH 7.6).

4    Sections were subjected to pepsin digestion (0.025% in 0.01M HCl) for 30 minutes at 37°C.

5    Sections were washed in TBS for 1 to 2 minutes;

6    Non-specific binding was inhibited using Toxoplasma antibody-free normal mouse serum (DAKO) diluted 1/5 in TBS at room temperature for 30 minutes;

7    Sections were rinsed in TBS for 1 to 2 minutes;

The sections for different studies were then treated either as:

8    sections were incubated overnight at 4°C with the primary layer comprising Toxoplasma antibody-free anti-human neurofilament protein (anti-HNF, clone 2F11, Monosan) at the predetermined optimal dilution of 1/100;

9    Sections were washed as in step 3.

10    Sections were incubated at room temperature for 30 minutes with a second layer comprising Toxoplasma antibody-free, rabbit anti-mouse IgG (Dako) diluted 1/50 with TBS;

11    Sections were washed as in step 3.

12    Sections were incubated at room temperature with the third layer comprising Toxoplasma antibody-free, mouse PAP complex (Dako) diluted 1/100 with TBS.

13    Sections were washed as in step 3.

14    Sections were incubated with diaminobenzidine (DAB 0.5mg in 0.5ml dimethyl formamide made up to 10 ml with TBS, 0.5ml hydrogen peroxide was added and the solution filtered directly onto the sections) for 10 minutes;

15    Sections were washed as in step 3.

16    Sections were lightly stained with Mayer's haemalum dehydrated in IPA, cleared in xylene and mounted in XAM.


## 2.13.2.    PEROXIDASE-ANTI-PEROXIDASE PROTOCOL FOR FRESH FROZEN SECTIONS

For the immunocytochemical demonstration of lymphocyte subsets and macrophages the following panel of rat-anti-mouse monoclonal antibodies were used.

| T Lymphocytes | Epitope specificity |
|---|---|
| LY1. | CD8. |
| LY2. | CD5. |
| **B Lymphocytes** | |
| LR1 | CD19. |
| **Macrophages** | |
| MAC1 | CD14. |

The monoclonal antibodies against T cell and macrophages were kindly donated by Dr Roger James from the Department of surgery University of Leicester. The pan B cell marker was obtained from Serotec.

Exhaustive tests using the staining protocol described in section 2.13.1 for paraffin processed sections proved unsuccesful. Modifications to the digestion and blocking stages (steps 2,3 and 4 in section 2.13.1) proved equally unsatisfactory.

As a result of this preliminary work, attempts to use paraffin section for this study were abandoned and fresh frozen cryostat sections were used throughout.

Brain tissue for this experiment was prepared as described in section 2.6.

Fresh frozen sections (8um thick) were cut using a SLEE cryostat (SLEE medical) at approximately $-20^{\circ}C$, they were air dried and stained using the following protocol.

1    Endogenous peroxidase activity was blocked by immersing sections for 20 minutes in a solution of 0.5% hydrogen peroxide in methanol.

2    Sections were washed in two changes, 10 minutes each, of Tris buffered saline (TBS, pH 7.6).

3    Sections were washed in TBS for 1 to 2 minutes;

4    Non-specific binding was inhibited using Toxoplasma antibody-free normal mouse serum (DAKO) diluted 1/5 in TBS at room temperature for 30 minutes;

5    Sections were rinsed in TBS for 1 to 2 minutes; sections were incubated overnight at $4^{\circ}C$ with the primary layer comprising Toxoplasma antibody-free rat anti-mouse monoclonal antibodies from the panel described above at the predetermined optimal dilution of between 1/50 and 1/200.

6    Sections were washed as in step 2.

7    Sections were incubated at room temperature for 30 minutes with a second layer comprising Toxoplasma antibody-free goat anti-rat IgG (Dako) diluted 1/50 with TBS;

8    Sections were washed as in step 2.

9    Sections were incubated at room temperature with the third layer comprising Toxoplasma antibody-free rat PAP complex (Dako) diluted 1/100 with TBS.

10    Sections were washed as in step 2.

11    Sections were incubated with diaminobenzidine (DAB 0.5mg in 0.5ml dimethyl formamide made up to 10 ml with TBS, 0.5ml hydrogen peroxide was added and the solution filtered directly onto the sections) for 10 minutes;

12    Sections were washed as in step 2.

13    Sections were lightly stained with Meyer's haemalum dehydrated in IPA, cleared in xylene and mounted in XAM.

## 2.14.    IMMUNOCYTOCHEMISTRY ELECTRON MICROSCOPY

### 2.14.1.    Preparation of sections

Mice were anaesthetised by carbon dioxide inhalation and perfused first with warmed heparinized isotonic saline, then whole body perfused with Karnovsky's formaldehyde-gluteraldehyde fixative (Karnovsky, 1966). Brains were then removed and immersed in fresh Karnovsky's fixative for not more than 4 hours at $4^{\circ}C$. Selected blocks of tissue of less than $1mm^3$ were processed without osmication, into the hydrophilic acrylic resin L.R. White (London Resin Company). This was achieved by partially dehydrating the specimens with 70% ethanol for 30 minutes followed in infiltration with a 50:50 mixture of L.R. White and 70% ethanol for 1 hour, followed by two changes of neat L.R. White resin at $37^{\circ}C$. Polymerization was achieved by maintaining blocks under anaerobic conditions at $50^{\circ}C$ for 20 hours, in a standard microbiological anaerobic jar with vanadium pentoxide catalyst and activated Oxoid Gas Pack (TM). Semithin sections were cut on an ultramicrotome with a glass knife and stained with 1% toluidine blue in 1% borax. Ultrathin sections of blocks containing intact Toxoplasma tissue cysts were cut and mounted onto 200 mesh Formvar-coated nickel grids.

### 2.14.2.    PRODUCTION OF ANTI-TOXOPLASMA ANTIBODY

Two NZW rabbits (Supplied by Fisons Ltd) of approximately 2.5 kg body weight and seronegative for Toxoplasma antibodies using the direct agglutination test as

described in section 2.7., were used to raise anti-
Toxoplasma antiserum. The method used was that described by
Hay et al. (1985). The rabbits were injected intravenously
with a suspension of saline-washed formalin-killed
endozoites of the RH strain of Toxoplasma on four
consecutive weeks. The parasites had been harvested from
the peritoneal exudate of mice using the method of Tryon et
al. (1978). The rabbits were then challenged on two
further occasions with live endozoites of the RH strain.
Fourteen days after the final challenge the rabbits were
anaesthetised and exsanguinated. Blood was incubated for
one hour at 37°C to produce serum which was then
centrifuged to remove cells and debris.

The IgG fraction of the pooled sera was isolated using a
single batch adsorption with DE-52 cellulose (Whatman)
employing the method of Reif (1969).


2.14.3.  **ELECTRON IMMUNOCYTOCHEMISTRY PROTOCOL**

The rabbit polyclonal anti-Toxoplasma IgG prepared as
described in section 2.14.2, was optimally diluted (1:200)
in Tris buffered saline (TBS, pH 7.6), which contained 1%
Toxoplasma antibody free, bovine serum albumen, and was
applied to sections containing intact Toxoplasma tissue
cysts. After washing in TBS, Toxoplasma-antibody free goat
anti-rabbit immunoglobulin at optimum dilution (1:50) in
TBS, labelled with 15nm colloidal gold particles was
applied to the sections. Sections were washed in TBS,
double-contrasted using uranyl acetate and lead citrate,
and examined using a JEOL 100CX TEMSCAN electron
microscope.

Specificity of the primary antibody preparation was determined by its omission from the immunocytochemical protocol.

All anti-sera and reagents were tested using the direct agglutination test for _Toxoplasma_ antibodies as described in section 2.7.

CHAPTER 3

RESULTS

## 3.1.    LIGHT MICROSCOPE HISTOCHEMISTRY OF INTACT TISSUE CYSTS

Twenty mice prepared as in section 2.5 were used in this study. Brain tissue was processed as described in section 2.6.

In rare instances only were tissue cysts observed to be obviously located within the soma of neurones (Figure 10 and Figure 11). In all other cases there appeared to be no obvious association with host cells (Figure 12). The tissue cyst "wall" was only weakly stained by the PAS method (Figure 13) and not at all by methenamine silver (Figure 14). The cystozoites, however, showed intense PAS and methenamine silver staining (Figure 13 and Figure 14 respectively). The Palmgren silver method demonstrated the characteristic argyrophilic nature of the cyst "wall" (Figure 15). When stained by the Bodian's protargol stain, intact tissue cysts showed intense staining of the cyst "wall" (Figure 16).

## 3..2.    ELECTRON MICROSCOPY OF INTACT TISSUE CYSTS

Twenty mice prepared as in section 2.5 were used in this study. Brain tissue was harvested as described in section 2.6.

At relatively low magnifications, electron microscopy revealed that the intact tissue cysts consisted of cystozoites which lay within an amorphous matrix, limited by a distinct tissue cyst "wall" (Figure 17). At higher magnification, this tissue cyst "wall" was seen to be composed of several recognisable components (Figure 18), and comprised an inner, intensely folded, unit membrane corresponding to the parasitophorous vacuole membrane

(Figure 19). Exterior to this membrane, a further layer could be seen which was separated by two unit membranes. Between these membranes were numerous microtubules aligned in parallel (Figure 20); these could also be seen in transverse section (Figure 21). Further increase in magnification allowed accurate measurement of these microtubules which showed an average outer diameter of 27nm. (Figure 22). Interspersed between these microtubules could be seen oval and elongated mitochondria (Figures 23 and 24). At the ultrastructural level no obvious host cell nucleus was observed within the wall of any tissue cysts examined. There was no evidence of axonal or dendritic processes associated with the parasitised cells. However, in all tissue cysts examined, at least one synaptic junction was seen between bouton terminals and the outer limiting membrane of the neurotubular layer corresponding to the host cell membrane (Figures 25 and 26).

In one poorly preserved sample, examination at the light microscopy level revealed a tissue cyst which was seen with what appeared to be a host cell nucleus associated with the tissue cyst "wall". Close examination at the ultrastructural level revealed that this nucleus belonged to a cell that was surrounding the outermost layer of an otherwise intact tissue cyst with obvious host cell components (Figure 27). The electron density of the plasma membrane of this cell, and the lack of cytoplasmic organelles, suggested that it was probably a Schwann cell (Figure 28).

Figure 10:    Semi-thin section of **Toxoplasma** infected
mouse brain. A mature tissue cust is seen
with what appears to be a host cell
nucleus (N).
Toluidine blue Mag X900.

Figure 11:    Toxoplasma infected mouse brain
showing host cell with developing
intracytoplasmic tissue cyst. The
morphology of the nucleus is
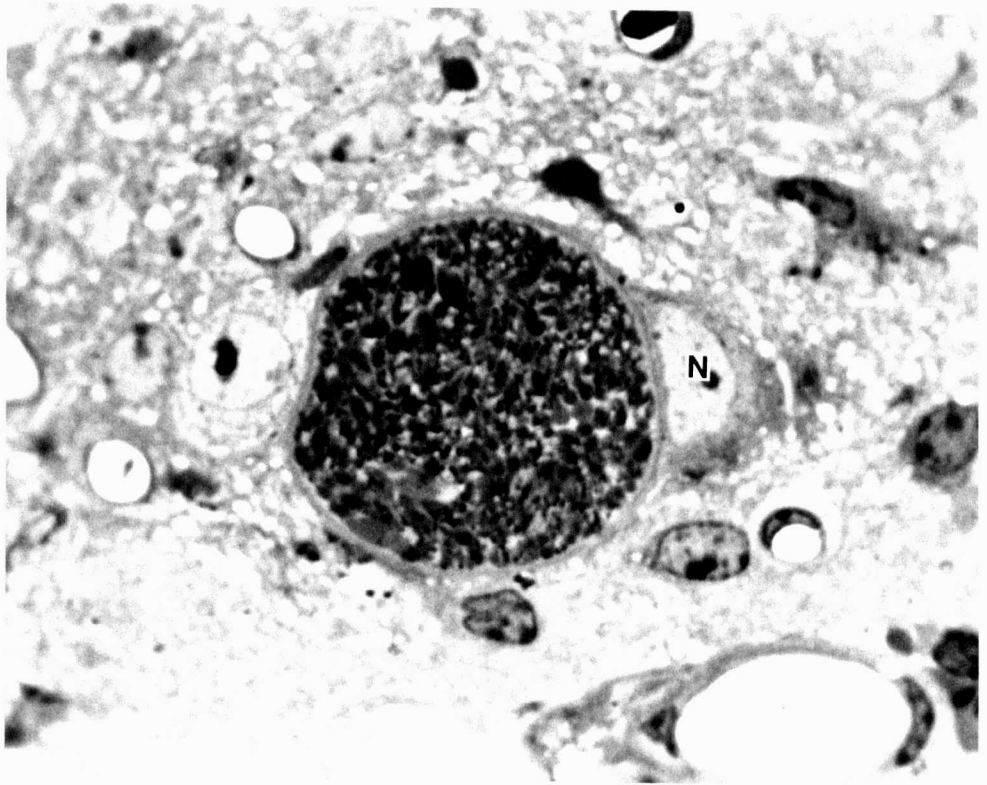strongly suggestive that the host
cell is neuronal.
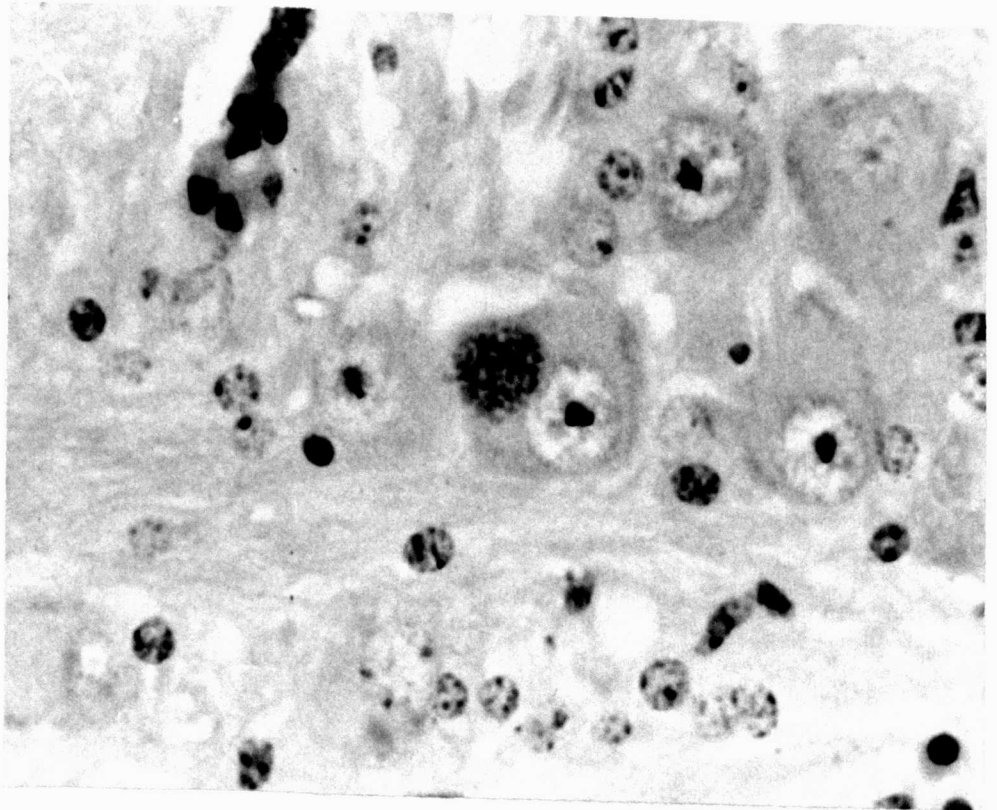(PAS Mag X 600)

49



Figure 10:



Figure 11:

Figure 12:     Toluidine blue stained section of
               _Toxoplasma_ infected mouse brain showing a
               well developed tissue cyst with no obvious
               host cell component within the cyst"wall"
               (Toluidine blue Mag x1000).

Figure 13:     _Toxoplasma_ tissue cyst in mouse brain
               stained with the PAS method. The cyst "wall"
               (W) shows only weak staining, whereas the
               cystozoites show intense PAS positive
               staining (PAS Mag. x1000)
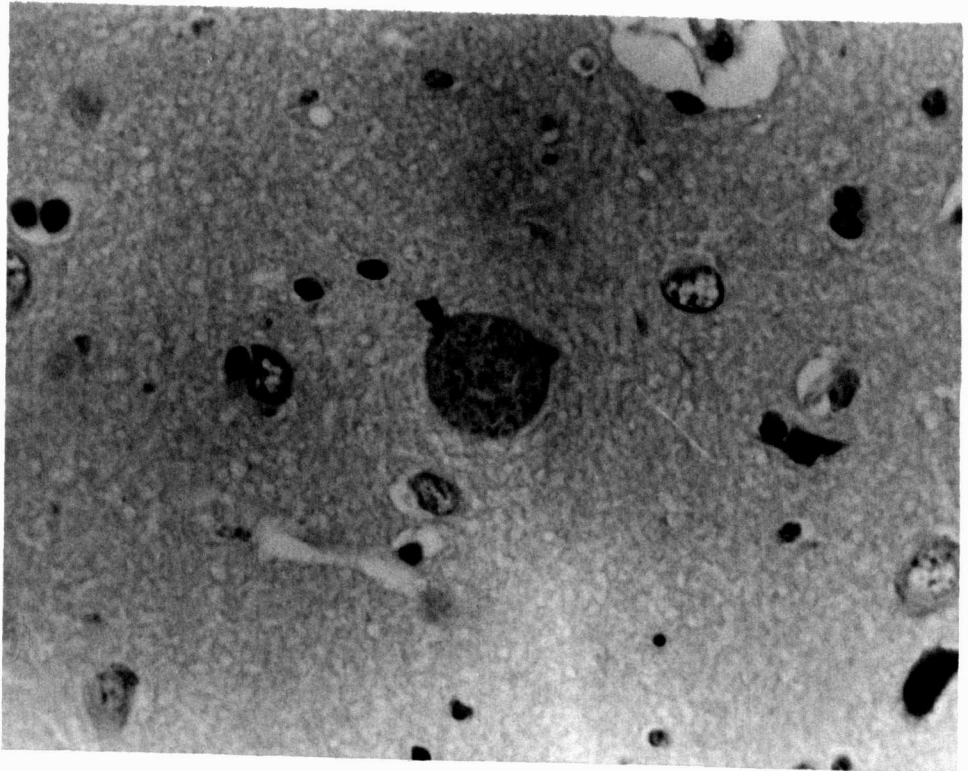
Figure 12:
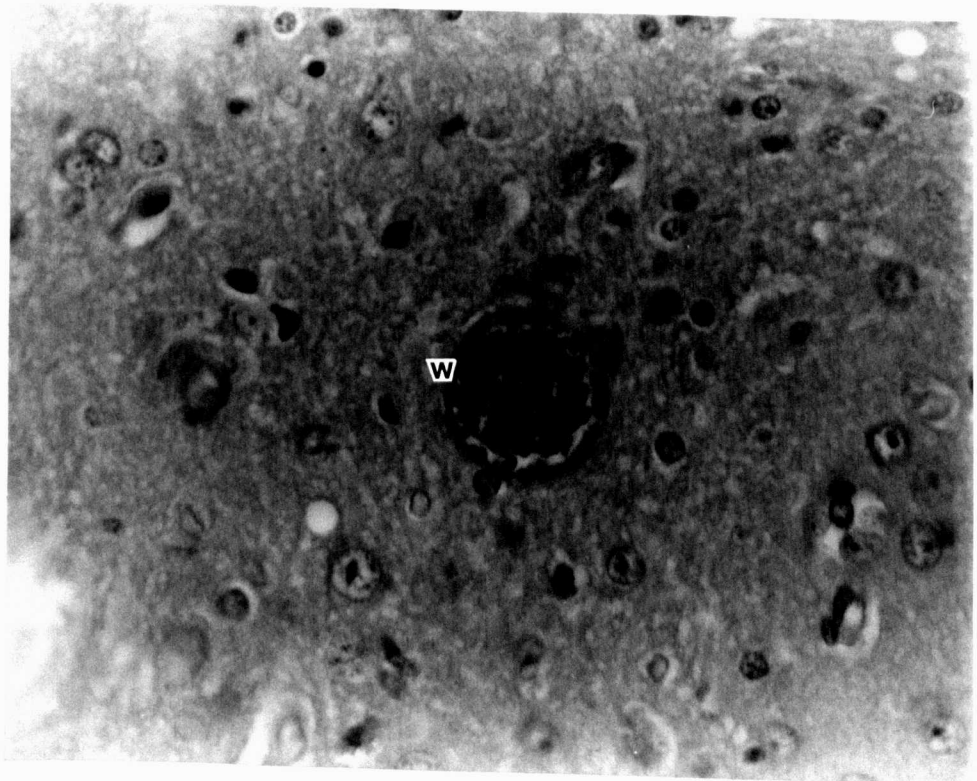


Figure 13:

**Figure 14:**    Mature <u>Toxoplasma</u> tissue cysts with
carbohydrate granules in the cystozoites
stained, but no staining of the tissue cyst
"wall" is seen.
(Hexamine Silver Mag. x1000).

**Figure 15:**    Mature <u>Toxoplasma</u> tissue cyst showing
argyrophilic nature of the tissue cyst
"wall"
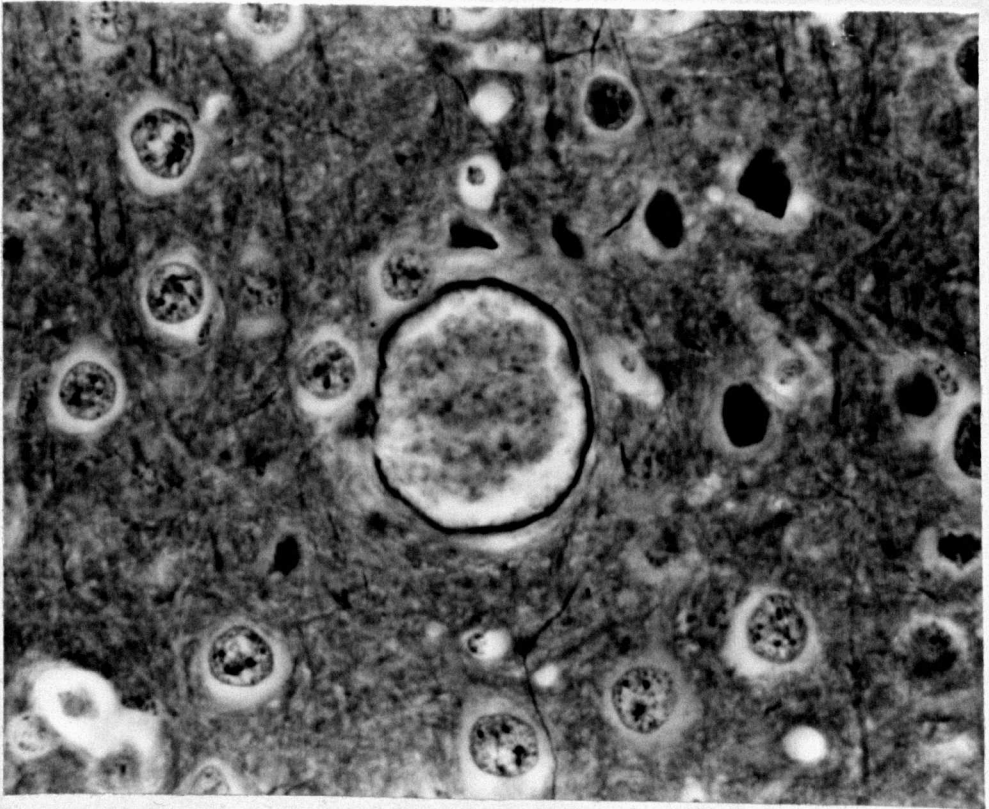(Palmgrens Mag. x1000)
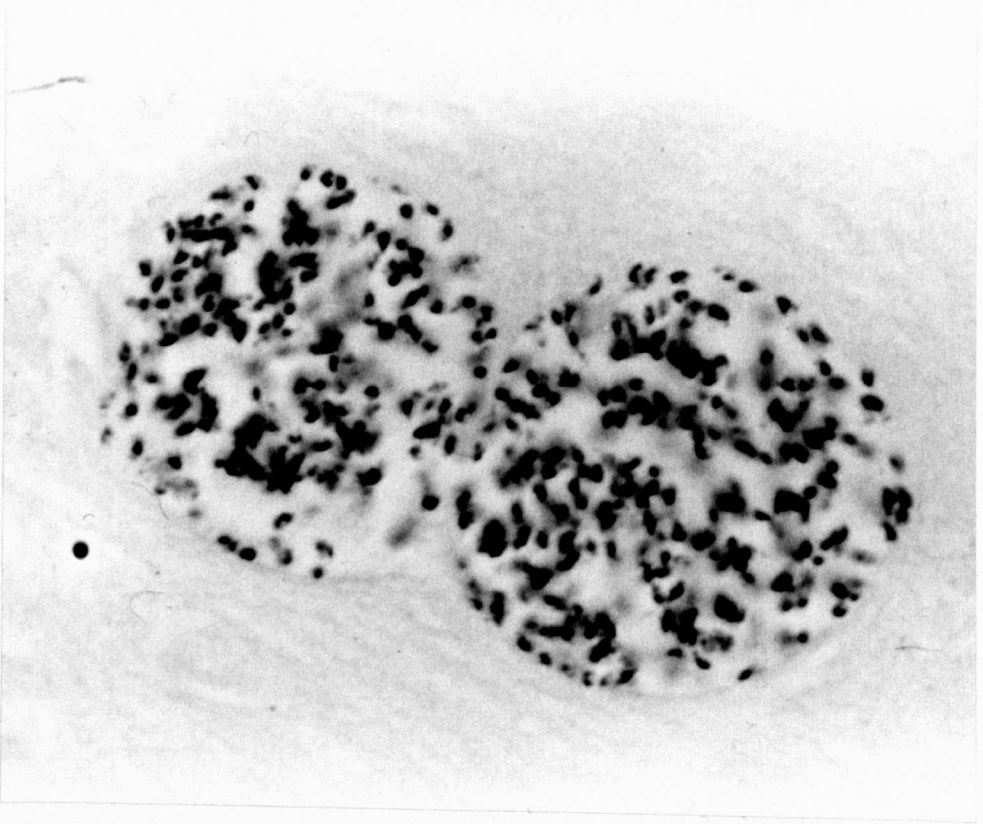
Figure 14:



Figure 15:

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**Figure 16:**   Mature tissue cyst showing cyst "wall"(W)
intensely impregnated with Protogol
silver.   (Bodian Mag. x1000).

**Figure 17:**   Low power electron micrograph showing
mature _Toxoplasma_ tissue cyst containing
numerous cystozoites (C) surrounded by a
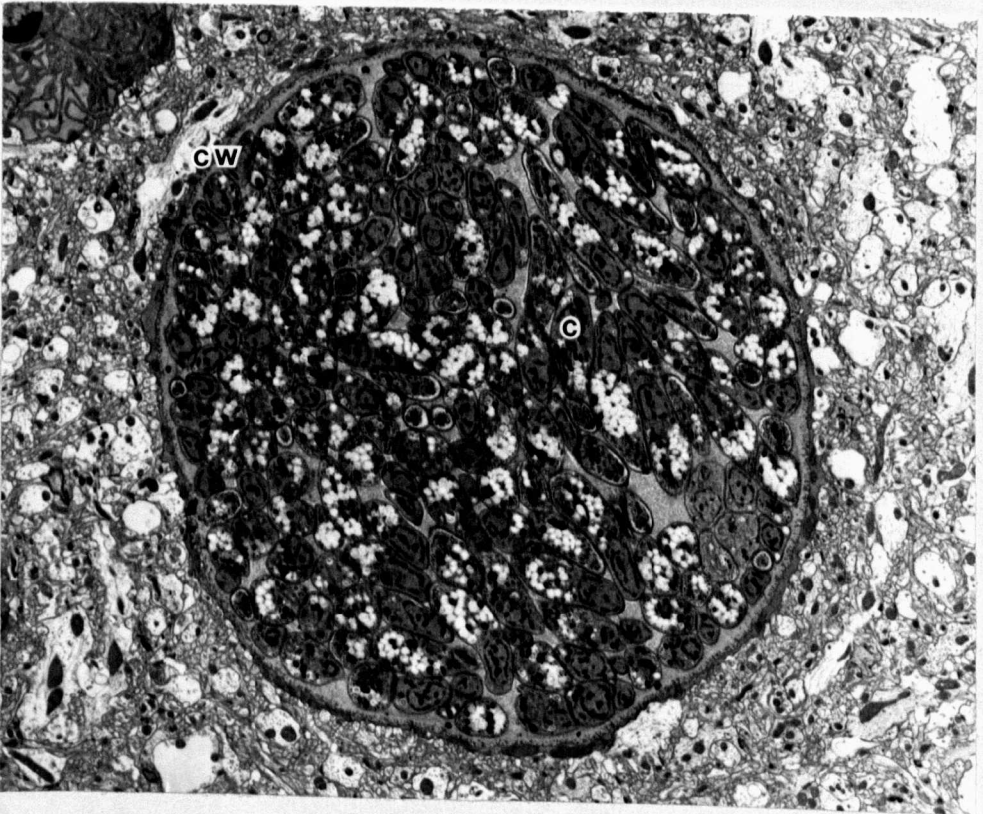tissue cyst "wall" (CW). (Mag. x3000)
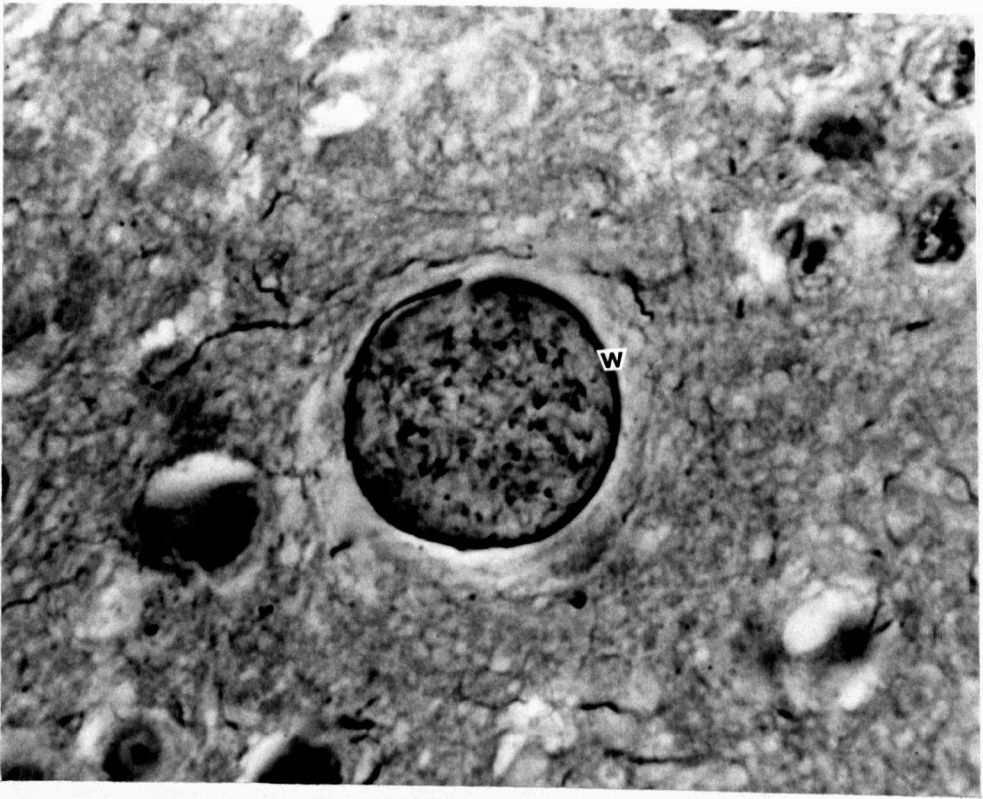
Figure 16:



Figure 17:

Figure 18:    Electron micrograph of the periphery of
              the cyst shown in Figure 17  Cystozoites (C)
              are enclosed within an inner convoluted and
              invaginated unit membrane, the
              parisitiphorous vacuole membrane (PCV) and
              an outer membrane bound layer of microtubule
              (MT) which are aligned in parallel.
              Mitochondria (M) are contained within the
              microtubular network. (Mag. x15000).

Figure 19:    Electron micrograph of the periphery of a
              tissue cyst similar to that shown in Figure
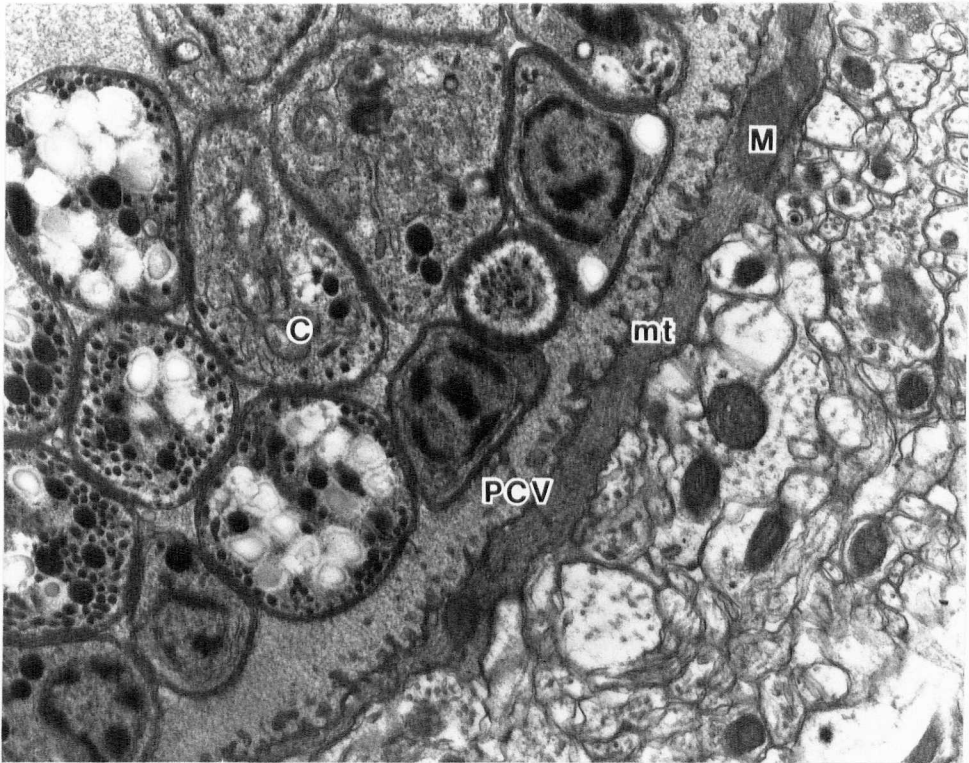              17.Microtubules (MT) are clearly seen.
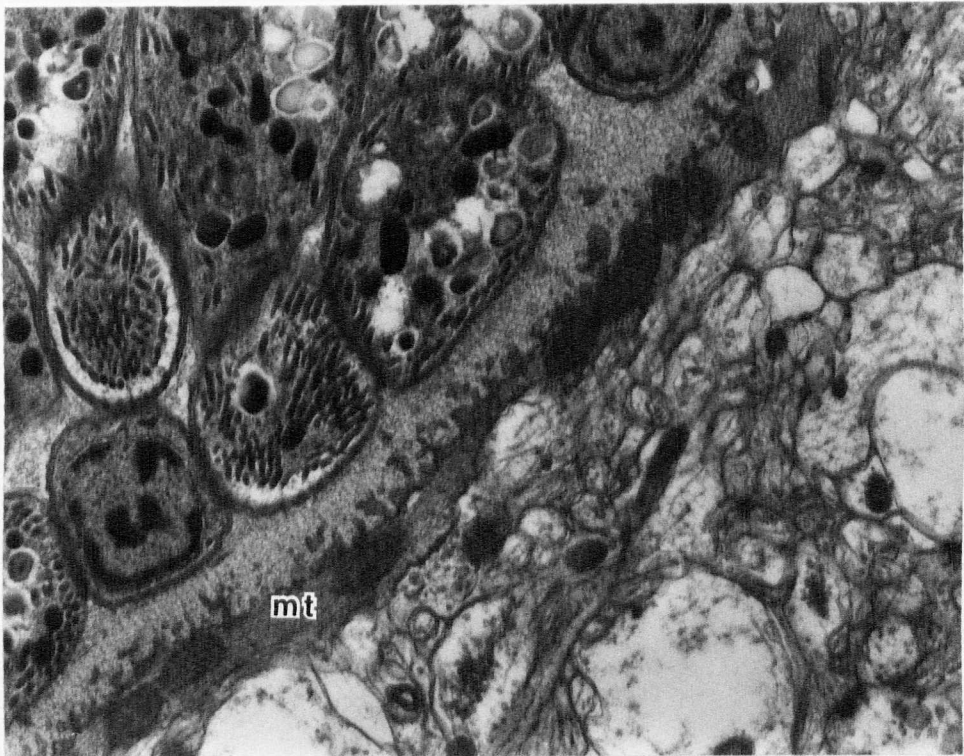              (Mag. x32000).

Figure 18:



Figure 19:

**Figure 20:**   Electron micrograph of the periphery of a
tissue cyst.   The inner parasitophorous
vacuole membrane (PCV) shows considerable
invagination; exterior to this, numerous
microtubules (MT) are clearly seen.
(Mag. x32000).

**Figure 21:**   Electron micrograph of a tissue cyst
"wall".   Microtubules (MT) are seen in
transverse section, together with rounded
mitochondria (M) enclosed within two plasma
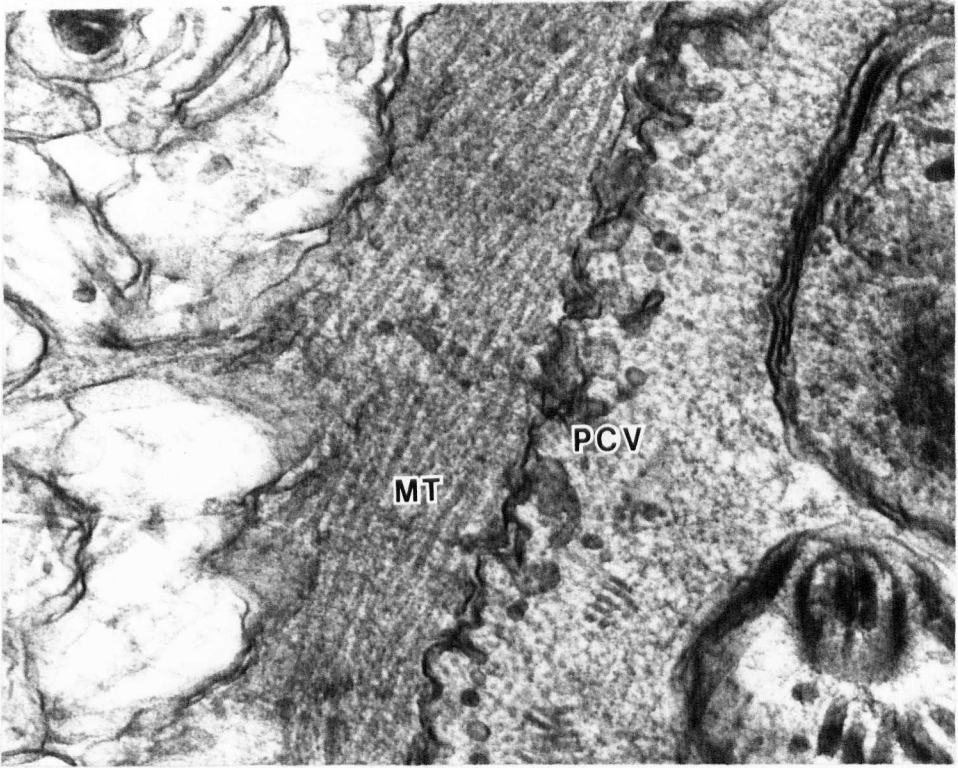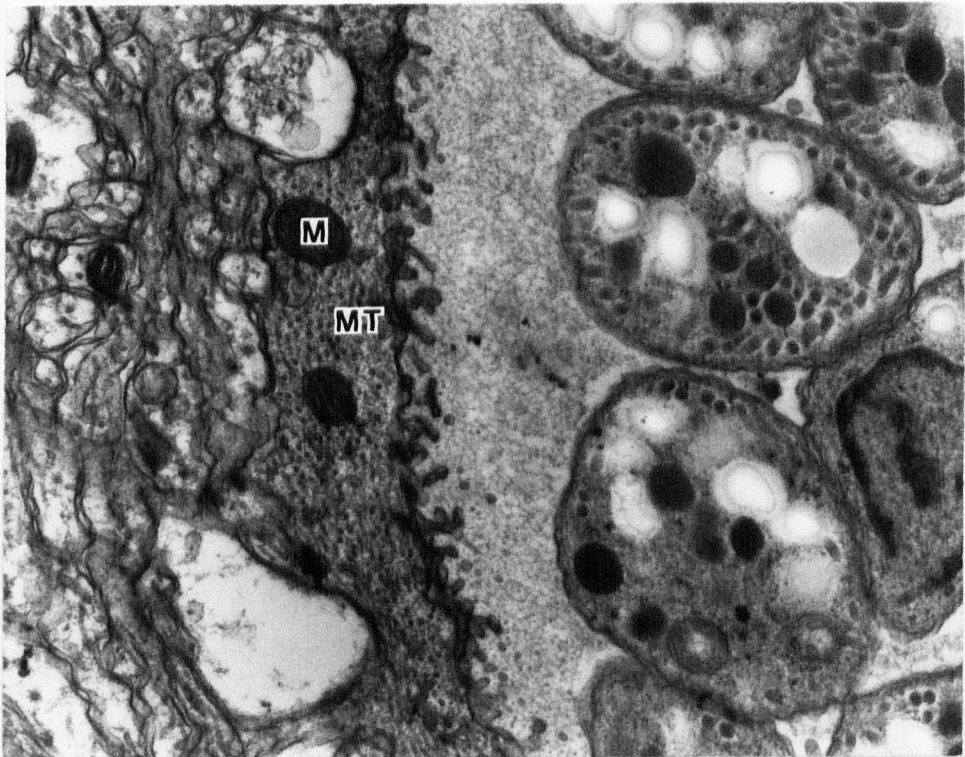membranes (arrow).
(Mag. x20000).

Figure 20:



Figure 21:

**Figure 22:**     High power electron micrograph of the
periphery of a tissue cyst "wall" clearly
showing the microtubular nature of the outer
component of the cyst wall.
(Mag. X 55000)

**Figure 23:**     Electron micrograph of the periphery of a
**Toxoplasma** tissue cyst "wall" showing
numerous elongated mitochondria (M)
interspersed between microtubules (MT)
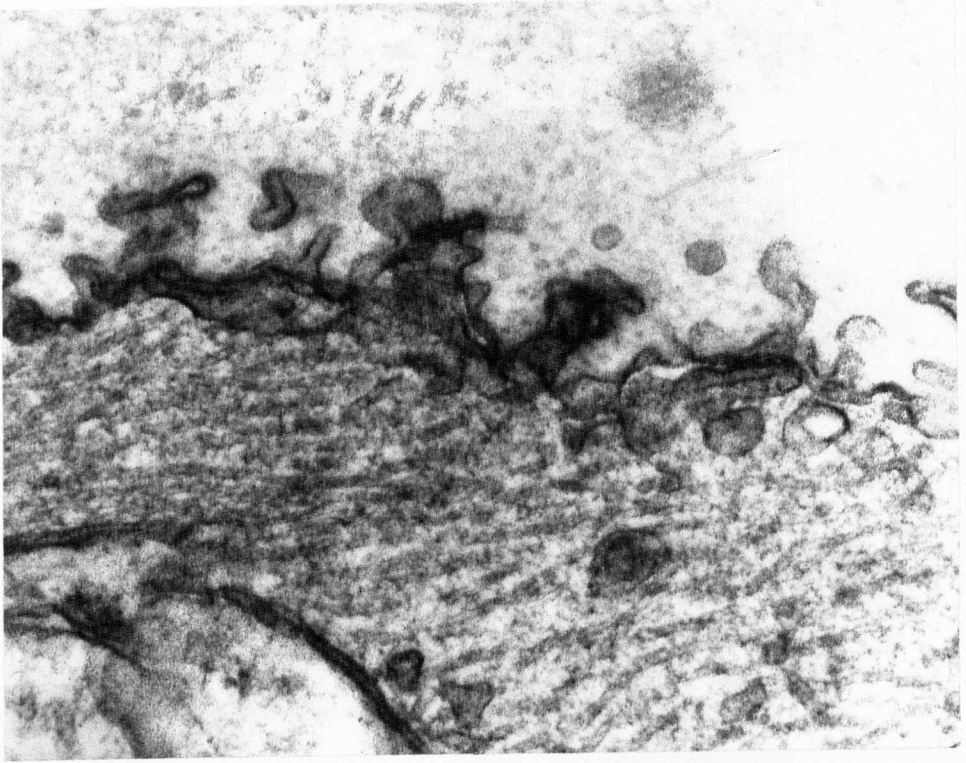enclosed within two plasma membranes.
(Mag. X 2100)

55



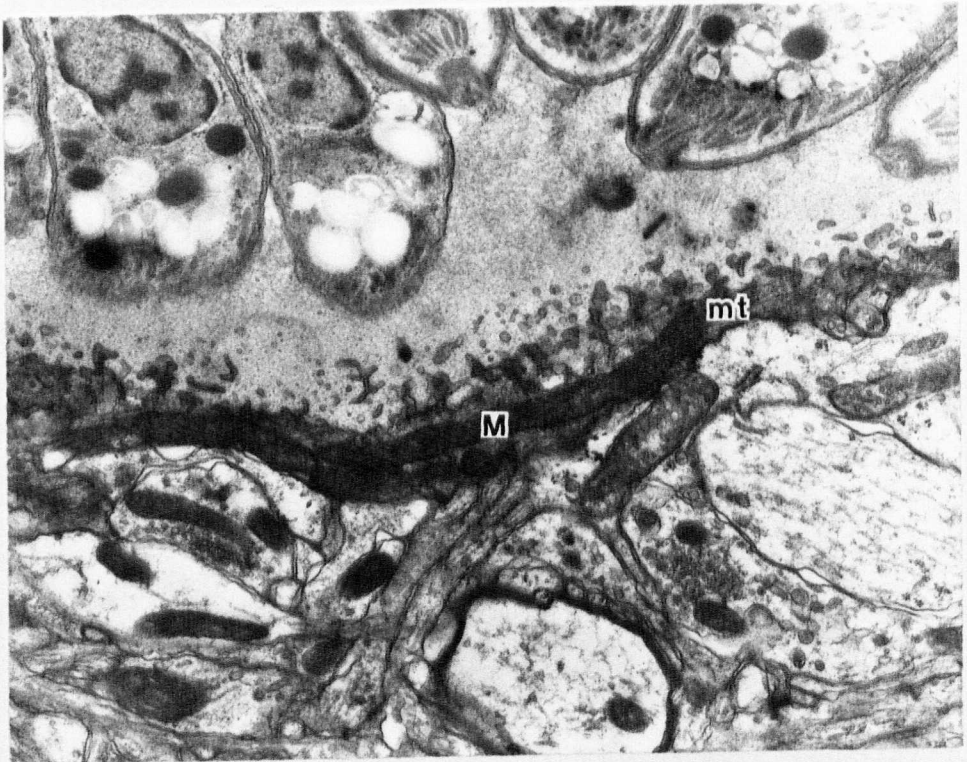Figure 22:



Figure 23:

Figure 24:     Electron micrograph of the periphery of a
               Toxoplasma tissue cyst showning oval
               mitochondria (M) and a parallel orientated
               microtubular layer (MT)
                 (Mag x 2100).

Figure 25:     Electron micrograph of the periphery of
               a tissue cyst "wall" showing a well-formed
               synaptic junction (S) on the outer unit
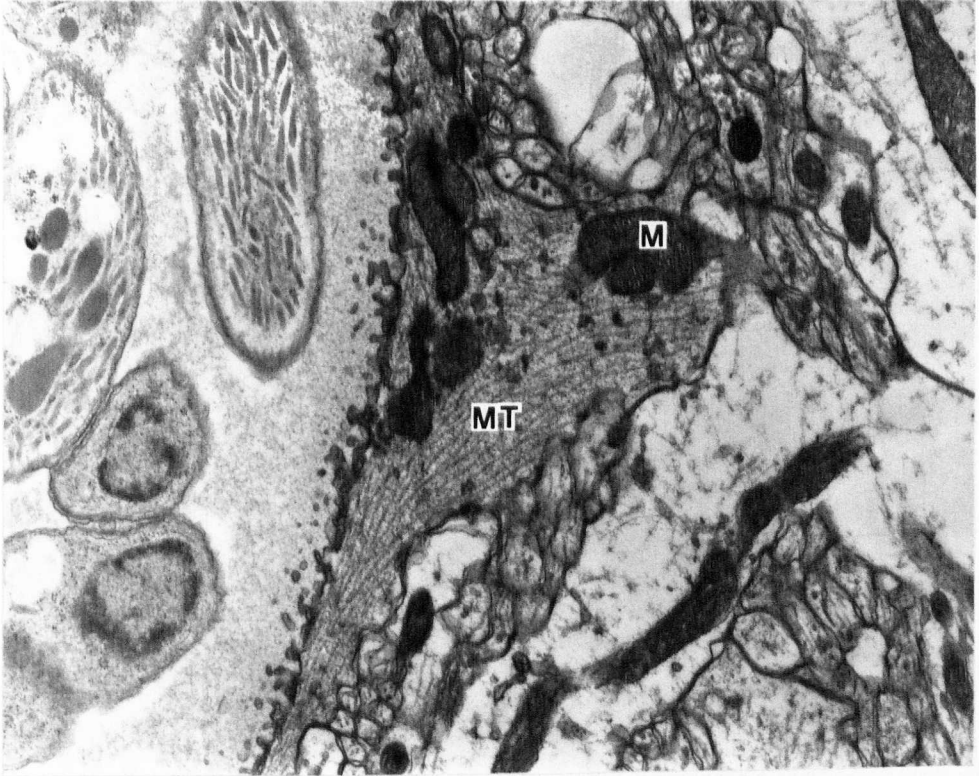               membrane of the microtubular layer (Mag.
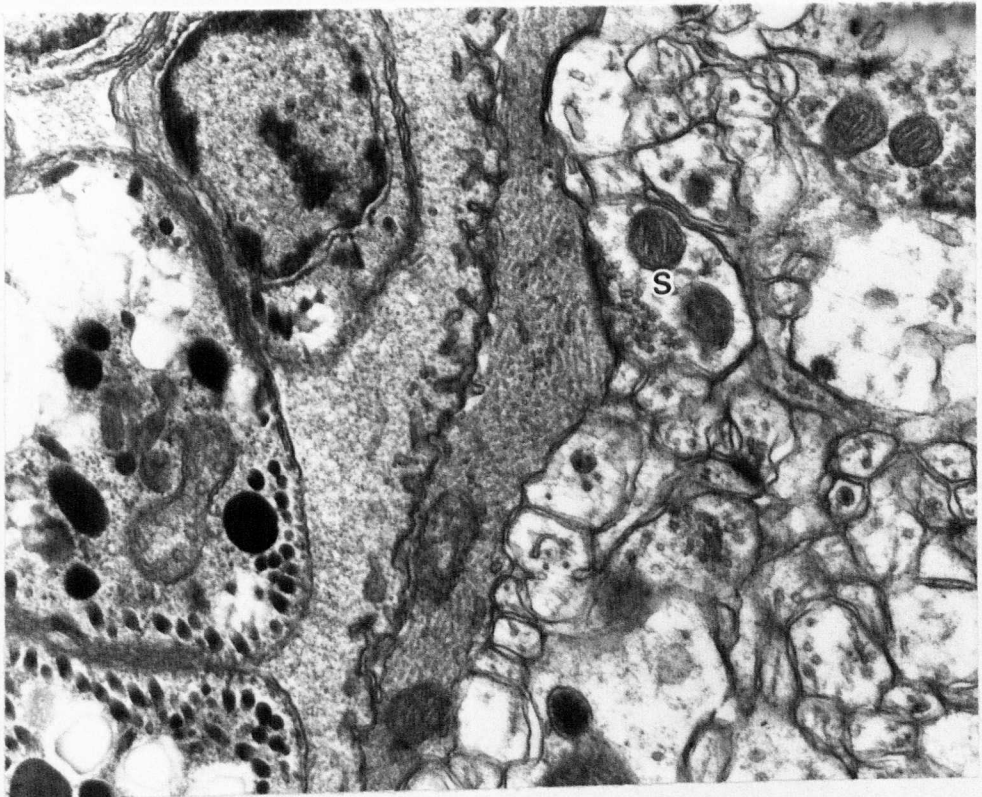               x28000).

Figure 24:



Figure 25:

**Figure 26:**    Electron micrograph of the periphery of a
                 tissue cyst similar to that shown in
                 Figure 24 showing well-formed synaptic
                 junction (S). (Mag X 33000)

**Figure 27:**    Low power electron micrograph of a mature
                 _Toxoplasma_ tissue cyst.  On the outer
                 surface can be seen what appears to be a
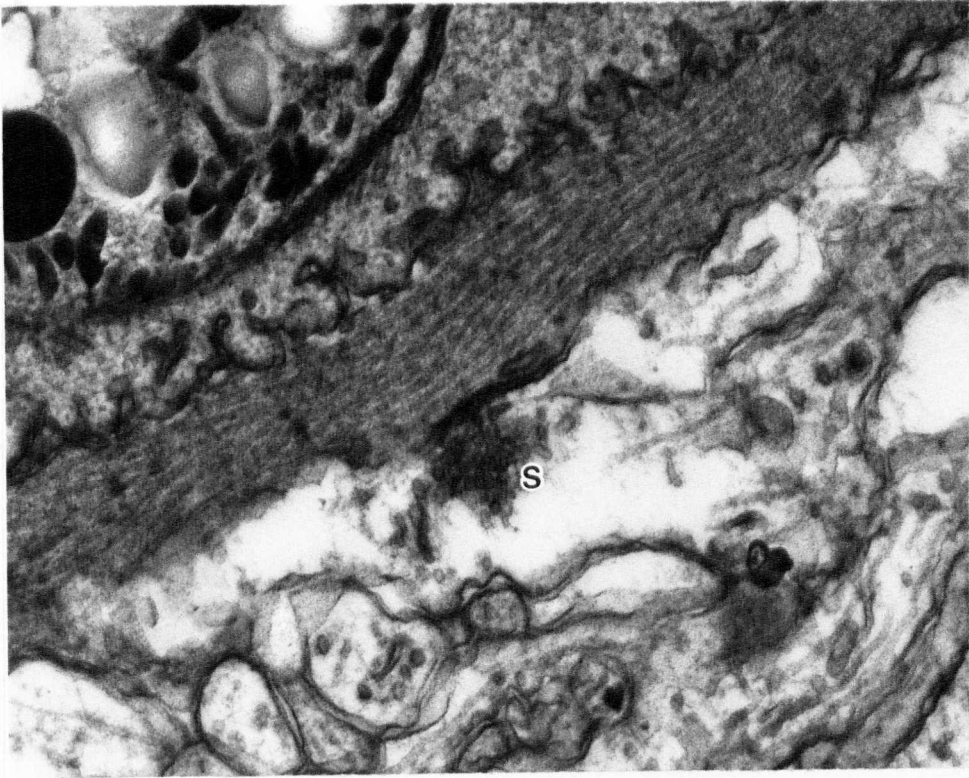                 host cell nucleus (N). (Mag. X3000).

Figure 26:



Figure 27:

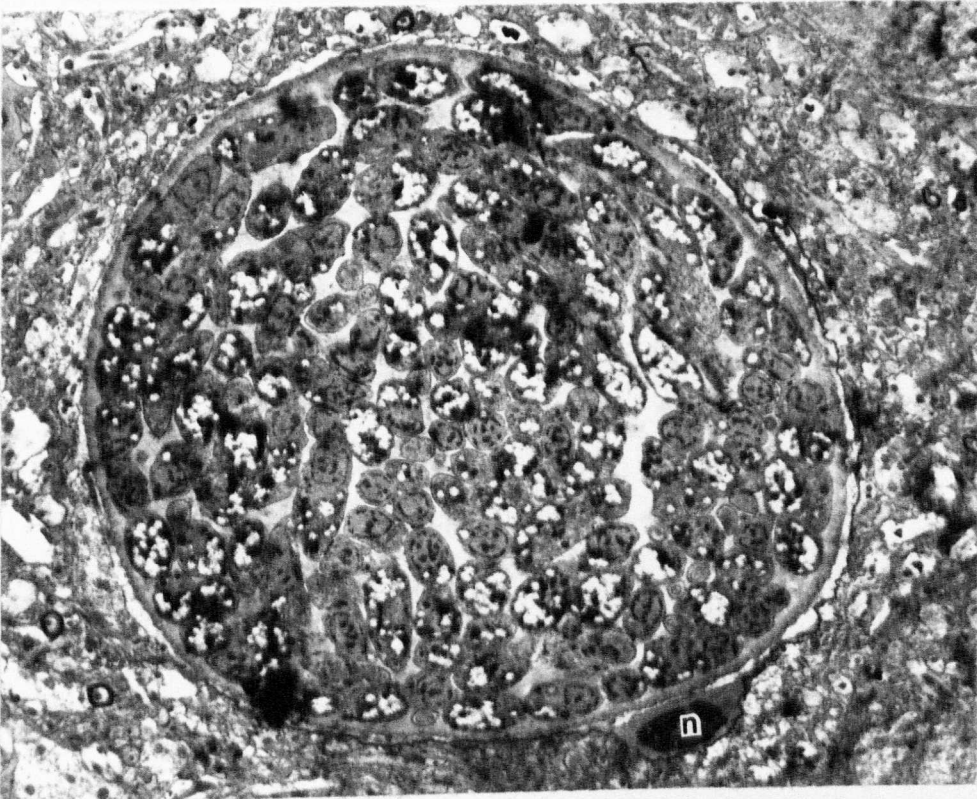**Figure 28:**     Higher power electron micrograph of the
nucleus and tissue cyst "wall" seen in
Figure 27  The cytoplasmic component is
devoid of organelles.  The outer plasma
membrane (arrow) shows intense electron
density.  Between the host cell cytoplasm
and the parasitophorous vacuole membrane
(PVC) can be seen a clear cytoplasm of true
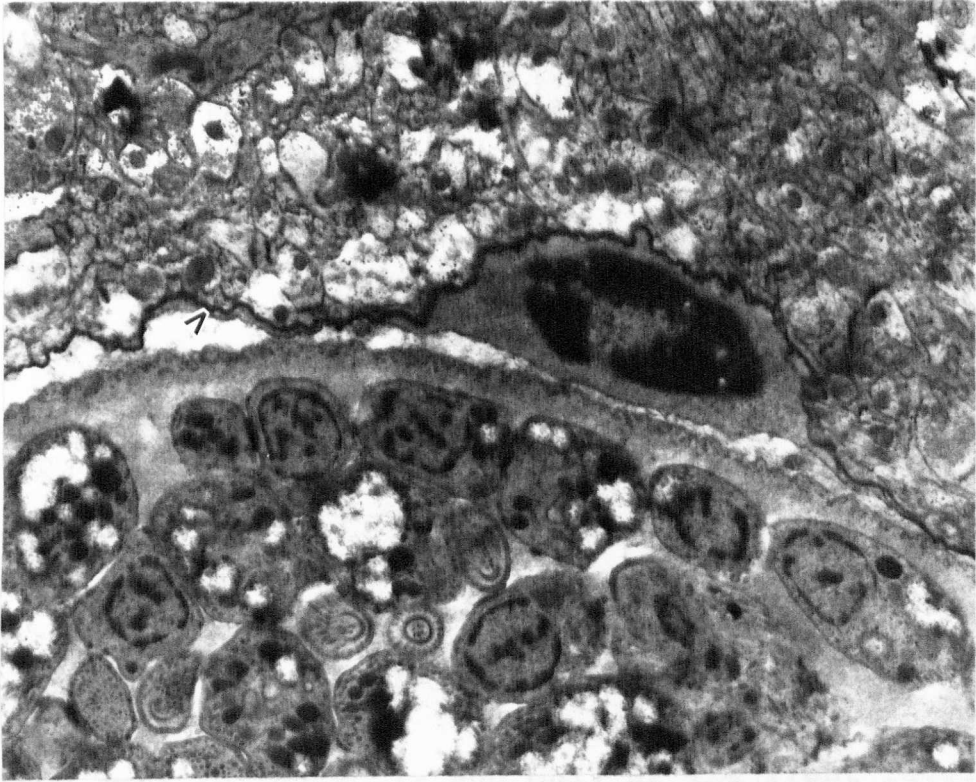host cell component of the cyst "wall".
(Mag x15000).

**Figure 28:**

## 3.3     HISTOLOGICAL CHARACTERIZATION OF INTACT TISSUE CYSTS

Twenty mice prepared as in section 2.5, were used in this study. Brain tissue was harvested as described in Section 2.6

### 3.3.1     MORPHOLOGICAL INVESTIGATIONS

The morphological appearance of intact tissue cysts within the brains of congenitally infected mice suggested that they were of two distinct categories.  The first category could be further subdivided into 2 types: those obviously contained within the soma of intact neurones (Figure 29), observed very infrequently and only at the light level, and another type which contained few cystozoites confined within a highly convoluted and invaginated parasitophorous vacuole membrane (Figure 30).  Exterior to this membrane, and contained within two distinct plasma membranes, was a component consisting of  thick band of microtubules orientated in parallel, among which were numerous interspersed mitochondria (Figure 31).  These two forms collectively comprised approximately 15% of all tissue cysts examined (22 of a total of 150).

The second category comprised tissue cysts which were much larger and which contained numerous tightly packed cystozoites (Figures 32 and 33).  The organisms were confined within a very flattened parasitophorous vacuole membrane which was considerably less invaginated than that observed in tissue cysts of the first category; the exterior component of the tissue cyst had a much thinner outer rim, and the interspersed mitochondria (Figures 34 and 35) were much more sparse.  This form of tissue cyst

comprised approximately 85% of all tissue cysts examined
(128 of a total of 150).

In all forms of tissue cyst observed, evidence of
innervation was noted in the form of synaptic plates on the
outer plasma membrane of the host component (Figure 36).

### 3.3.2 . **IMMUNOCYTOCHEMISTRY.**

The light microsope immunohistochemical demonstration of
neurofilament protein was carried out as described in
Section 2.13.1. Brains from twenty mice were prepared as in
Section 2.5 and tissue harvested as in Section 2.6 Results
showed that, irrespective of the morphological appearance
of the tissue cysts, a distinct band of staining could be
seen around the periphery of the tissue cysts (Figures 37
and 38). All tissue cysts examined from each of the brains
of the twenty mice, showed this pattern of staining In one
instance only, what appeared to be the host cell nucleus,
was observed in association with the neurofilament protein
stained layer (Figures 39 and 40). The specificity of the
neurofilament protein used in the PAP methodology was
demonstrated by its omission as the primary layer; this
resulted in no staining of the outer rim of the tissue
cyst. All neurones were stained using the complete PAP
protocol (Figure 41). In contrast neuroglial cells were not
stained using this protocol (Figure 42)

## 3.4.  ELECTRON IMMUNOCYTOCHEMISTRY OF INTACT TISSUE CYST

### 3.4.1 MORPHOLOGICAL ASPECTS

Twenty mice prepared as in Section 2.5 were used in this study. Brain tissue was harvested as described in Section 2.6.

Although _Toxoplasma_ antigen was distributed throughout the intact tissue cyst (Figure 43), it was especially concentrated in the vicinity of the inner aspect of the parasitophorous vacuole membrane (Figures 44 and 45).  The enclosed cystozoites were associated with smaller amounts of _Toxoplasma_ antigen relative to the matrix of the tissue cyst (Figures 46 and 47) This occurred irrespective of the location of the cystozoites within the tissue cyst. In some instances cystozoites appeared to be completely free of _Toxoplasma_ antigen (Figure 48).  Small amounts of _Toxoplasma_ antigen were observed in the host cell component of the intact tissue cyst and in the immediately adjacent neuropil (Figures 48,45 and 51).

When the anti-_Toxoplasma_ antibody was omitted from the immunocytochemical protocol described in Section 2.14.3, no _Toxoplasma_ antigen was detected with within the tissue cysts (Figure 52), or within the host cell components exterior to the parasitophorous vacuole membrane (Figures 50,51 and 52).

When the above immunocytochemical procedure (Section 2.14.3) was applied to brain tissue from mice uninfected with _Toxoplasma_ (as determined by negative serology and lack of tissue cysts in the brain), no colloidal gold deposition was detected.

Figure 29:     <u>Toxoplasma</u> tissue cyst within the cytoplasm of an intact neurone (Semithin toluidine blue Mag. X1000).

Figure 30:     Low power electron micrograph showing sparsely distributed cystozoites (C) contained within an amorphous matrix (M) bounded by a thickened outer layer of neurotubules with interspersed elongated mitochondria (M). (Mag. X5900)
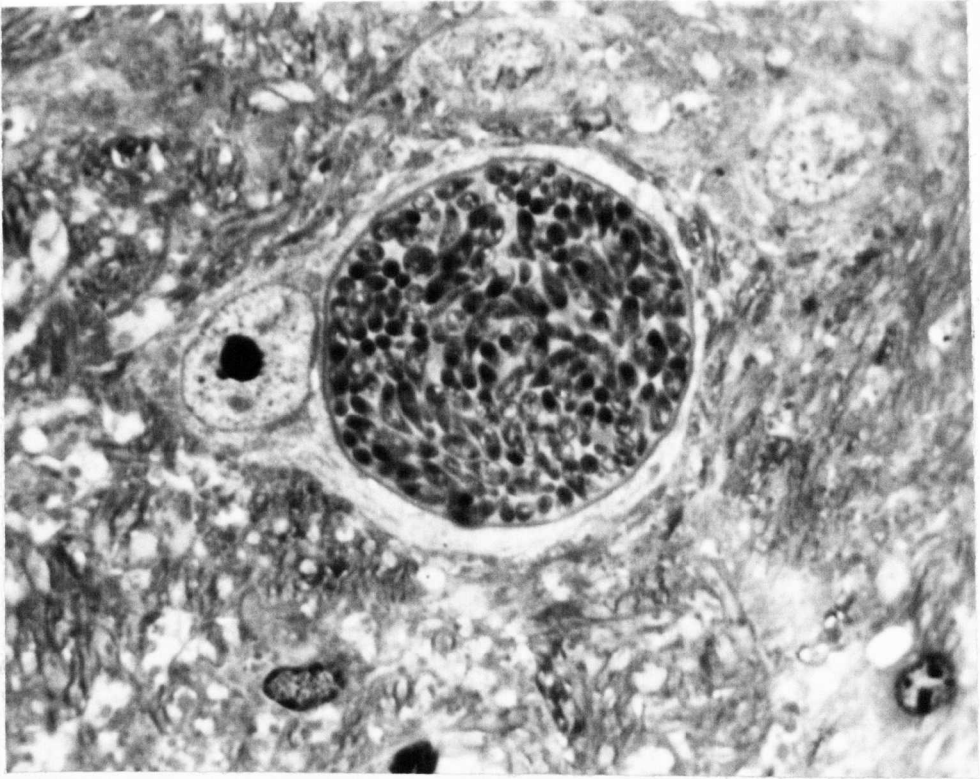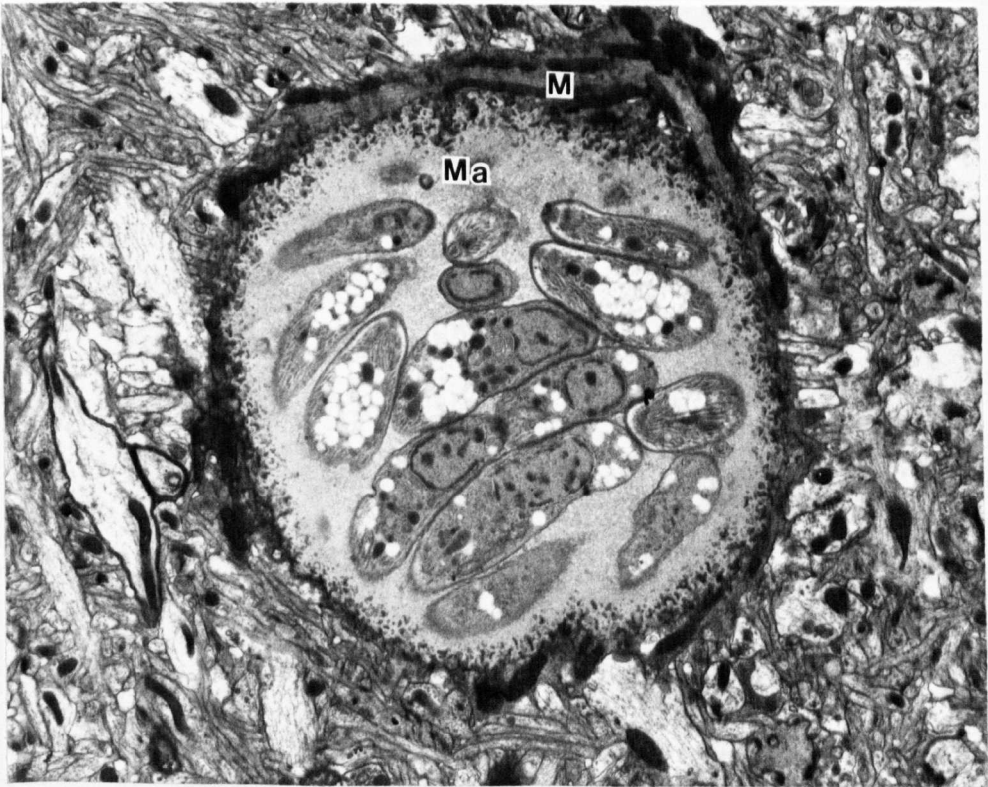
Figure 29:



Figure 30:

Figure 31:   Part of the cyst "wall" shown in
             Figure 29.  Cystozoites (C) and amorphous
             matrix (M) are contained within a highly
             convoluted inner membrane, the
             parasitophorous vacuole membrane (PCV),
             exterior to which is a membrane bound
             component comprising a thick band of
             microtubules (MT) orientated in parallel,
             containing numerous elongated mitochondria
             (Mag. x18200).

Figure 32:   Low power electron micrograph similar to
             Figure 27  showing densely packed
             cystozoites (C) contained within an
             amorphous matrix (M) bounded by a thin outer
             layer of microtubules with associated
             mitochondria (M).
             (Mag. x3500).
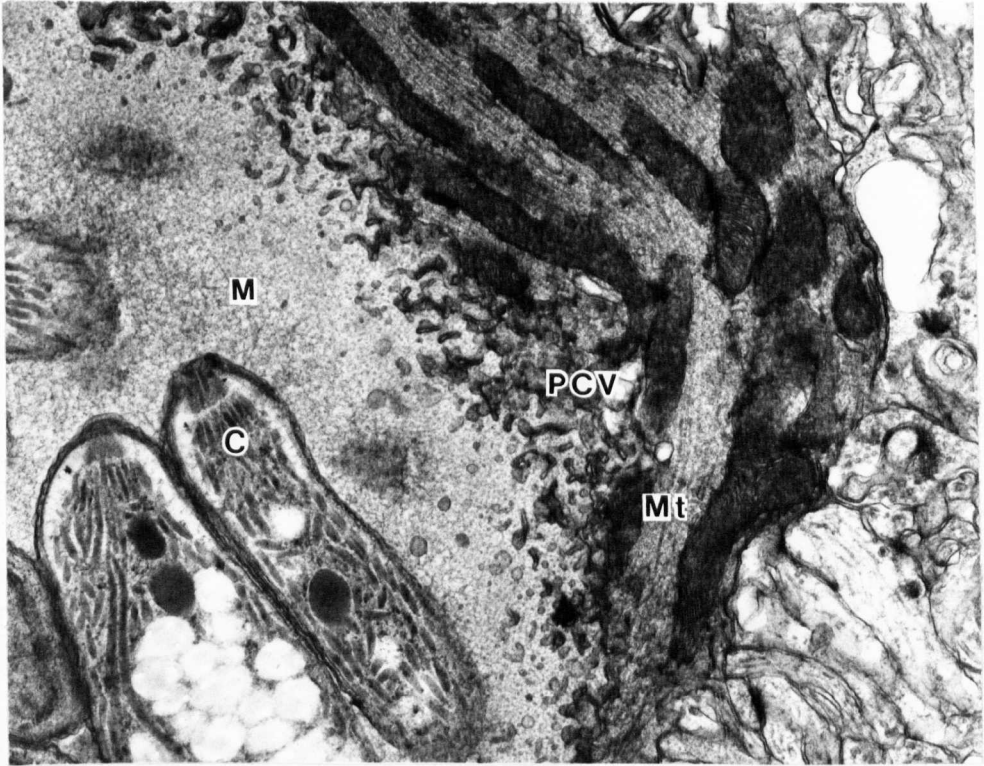
Figure 31:
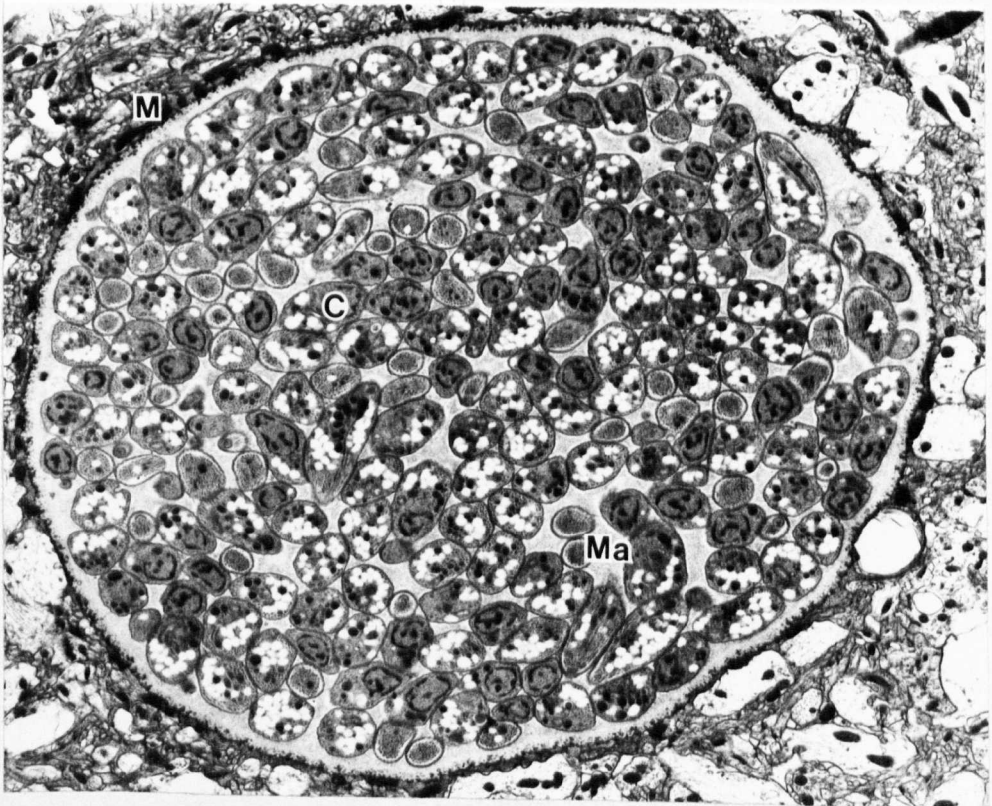


Figure 32:

Figure 33:     Low power electron micrograph showing
               features similar to Figures 27 and 32.
               (Mag. X3500).

Figure 34:     Part of the cyst shown in Figure 32.
               Cystozoites and amorphous matrix are
               contained within a moderately convoluted
               parasitophorous vacuole membrane (PCV),
               exterior to which is a thin band of
               microtubules orientated in parallel,
               containing few ovoid mitochondria.
               (Mag x21000).
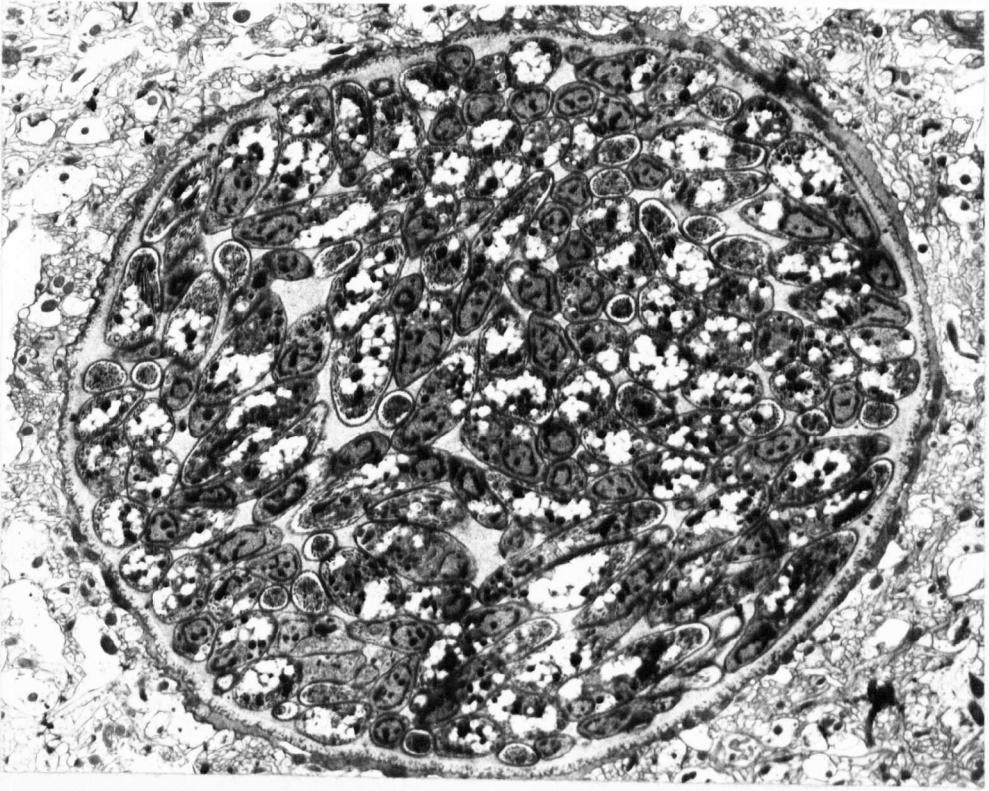
Figure 33:
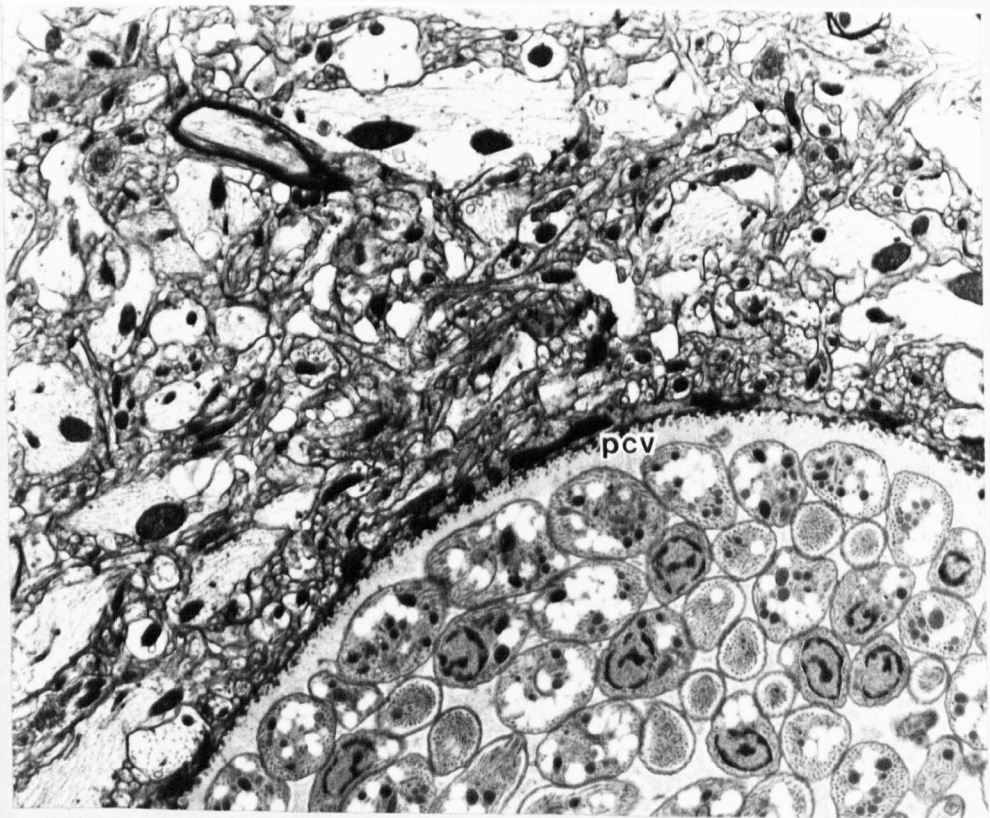


Figure 34:

**Figure 35:**     Part of tissue cyst shown in Figure 32.
showing marked reduction in the thickness of
the cyst "wall"..
(Mag x21000).

**Figure 36:**     Electron micrograph of periphery of a tissue
cyst showing well formed synaptic junction
(S) on the outer membrane of the host cell
component.
(Mag X30200).
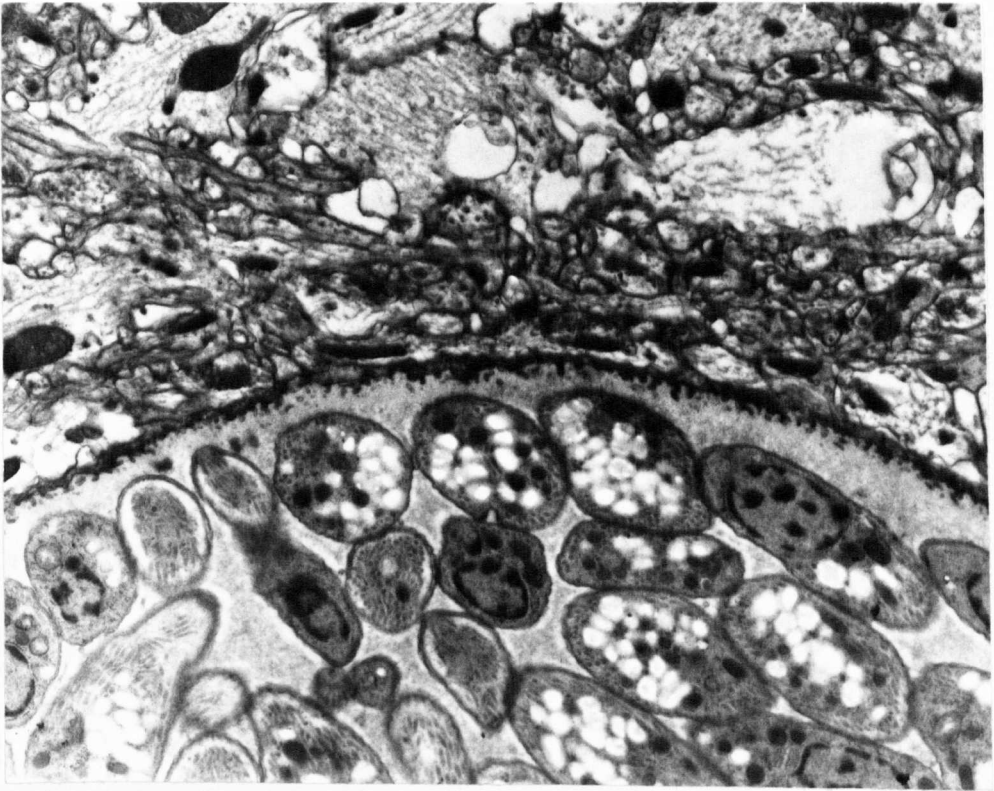
Figure 35:



Figure 36:

**Figure 37:**     <u>Toxoplasma</u> tissue cyst showing a distinct band of neurofilament protein (arrowed) which delimits the cystozoites from the host neurophil. (PAP/haemalum Mag. x1000).

**Figure 38:**     <u>Toxoplasma</u> tissue cyst showing features similar to Figure 37.
(PAP/haemalum Mag. x1000).
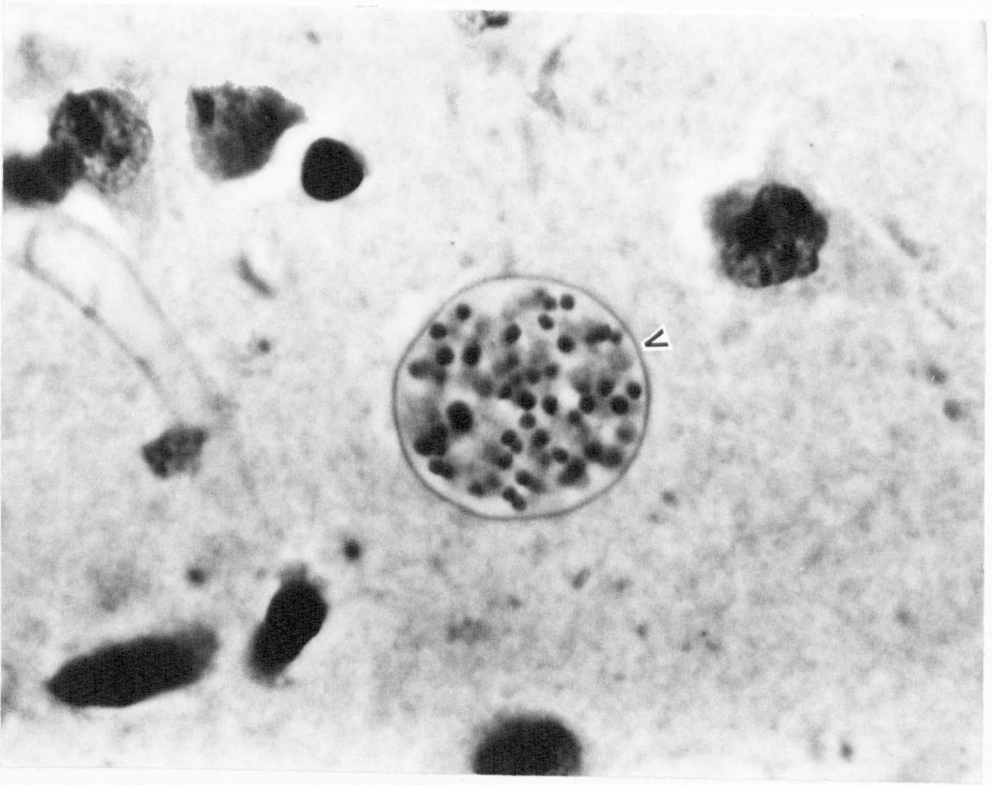
Figure 37:
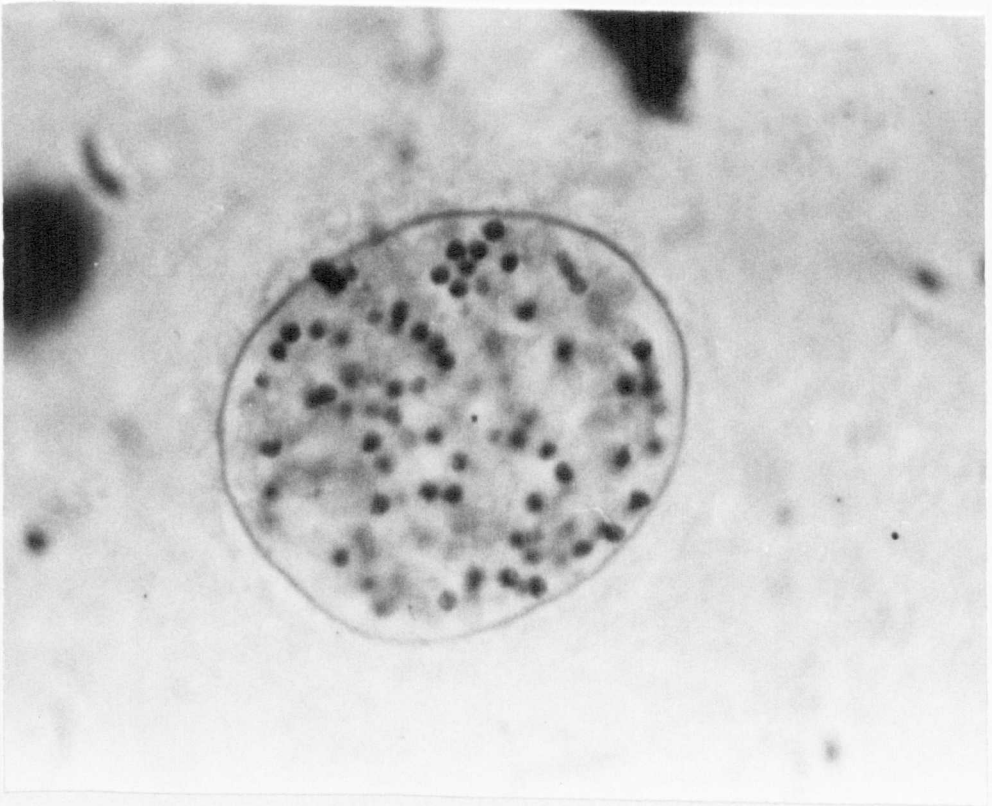


Figure 38:

Figure 39:      <u>Toxoplasma</u> tissue cyst showing a distinct
                band of neurofilament protein which delimits
                the cystozoites from the host neuropil and
                surrounds the host cell nucleus (N).
                (PAP/haemalum Mag.X1000).

Figure 40:      Two <u>Toxoplasma</u> tissue cysts seen in close
                proximity.  Both show neurofilament protein
                staining similar to Figure 36 and 37.
                (PAP/haemalum Mag.X1000).
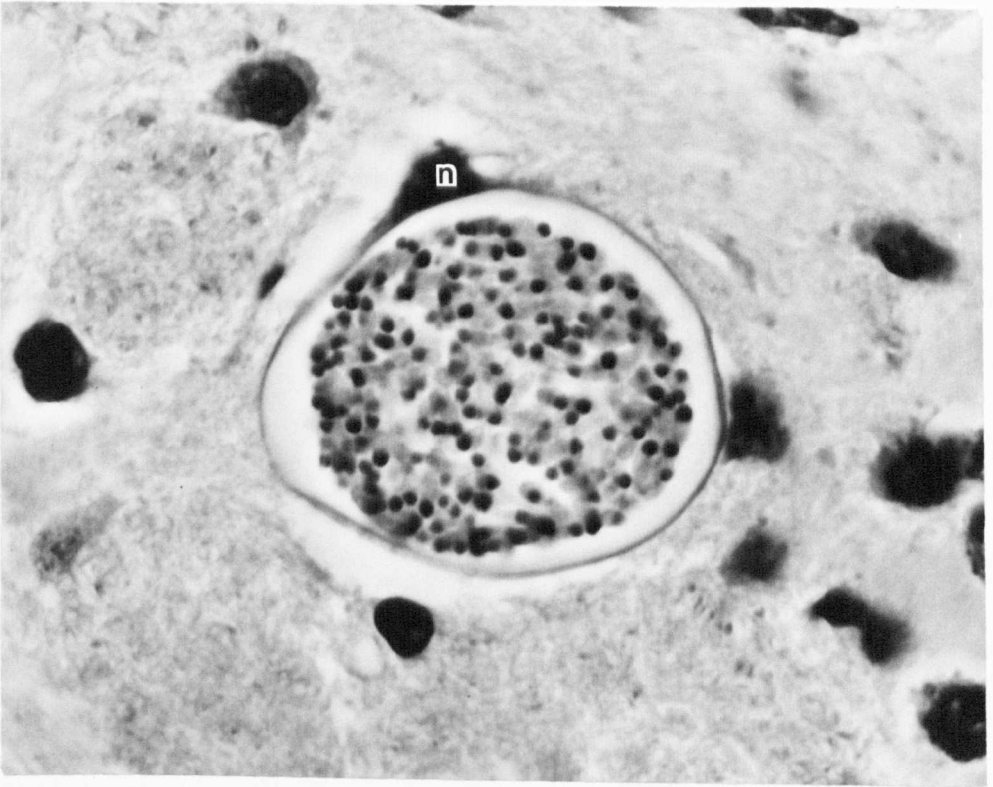
Figure 39:
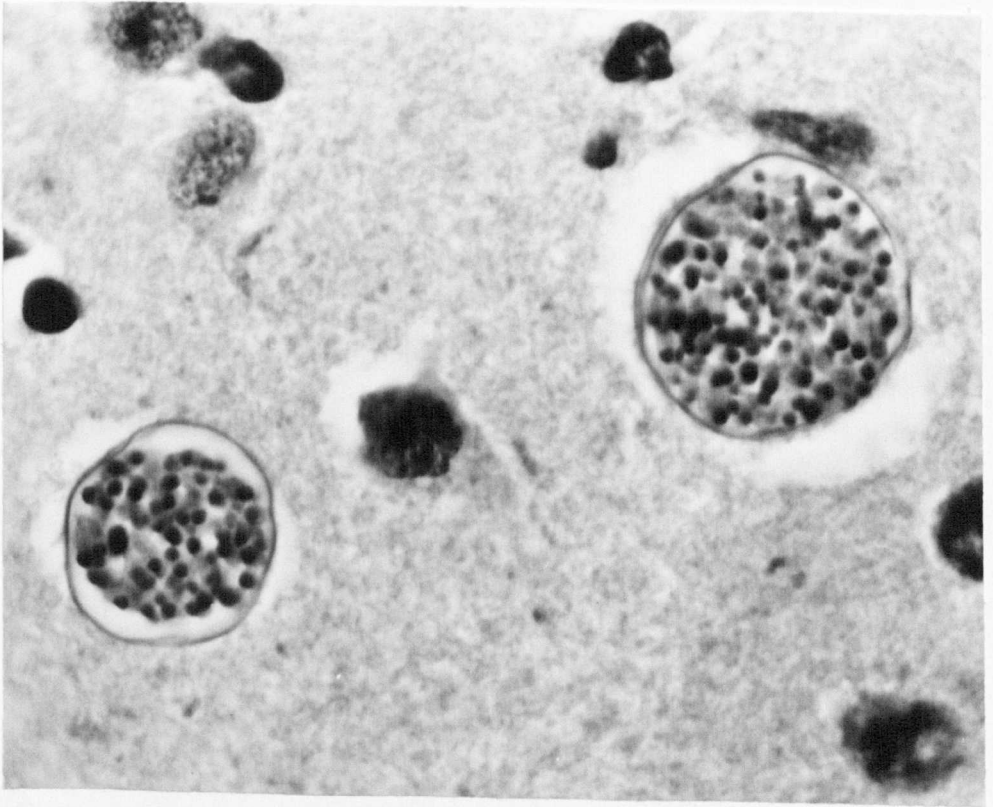


Figure 40:

**Figure 41:**     Bipolar neurone showing neurofilament
protein staining.
(PAP/haemalum Mag. x1000)

**Figure 42:**     Glial cell (G) from the brain of a mouse
congenitally infected with <u>Toxoplasma</u>
showing abscence of neurofilament protein
staining (PAP/Haemalum Mag X1000).

68
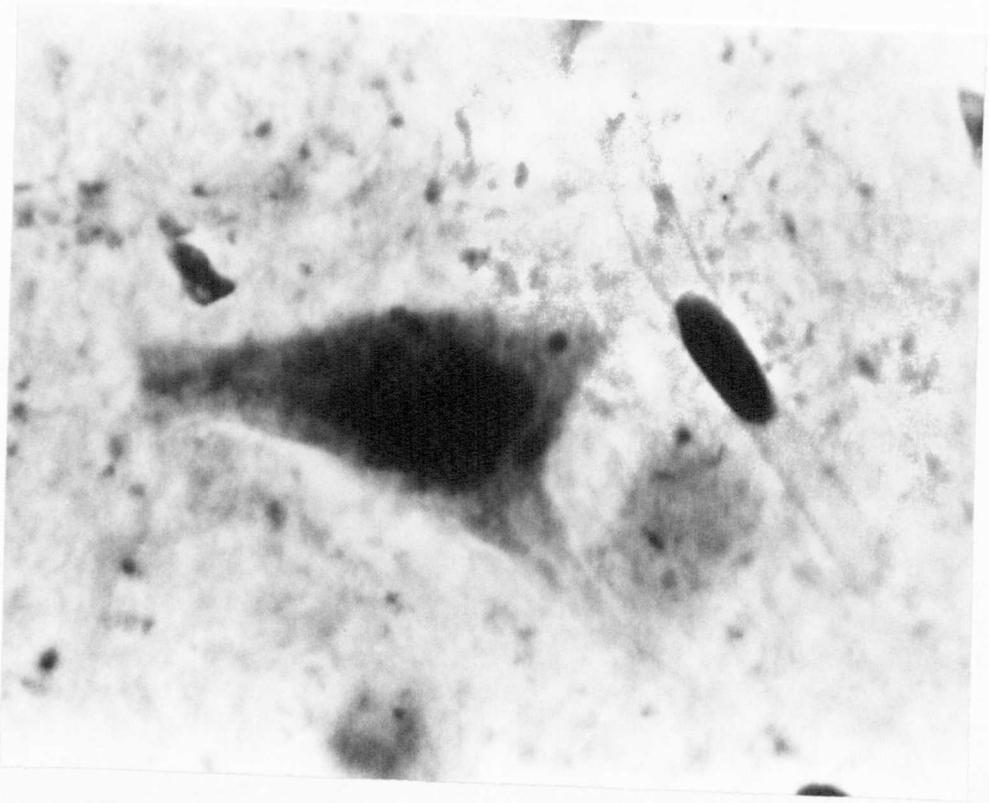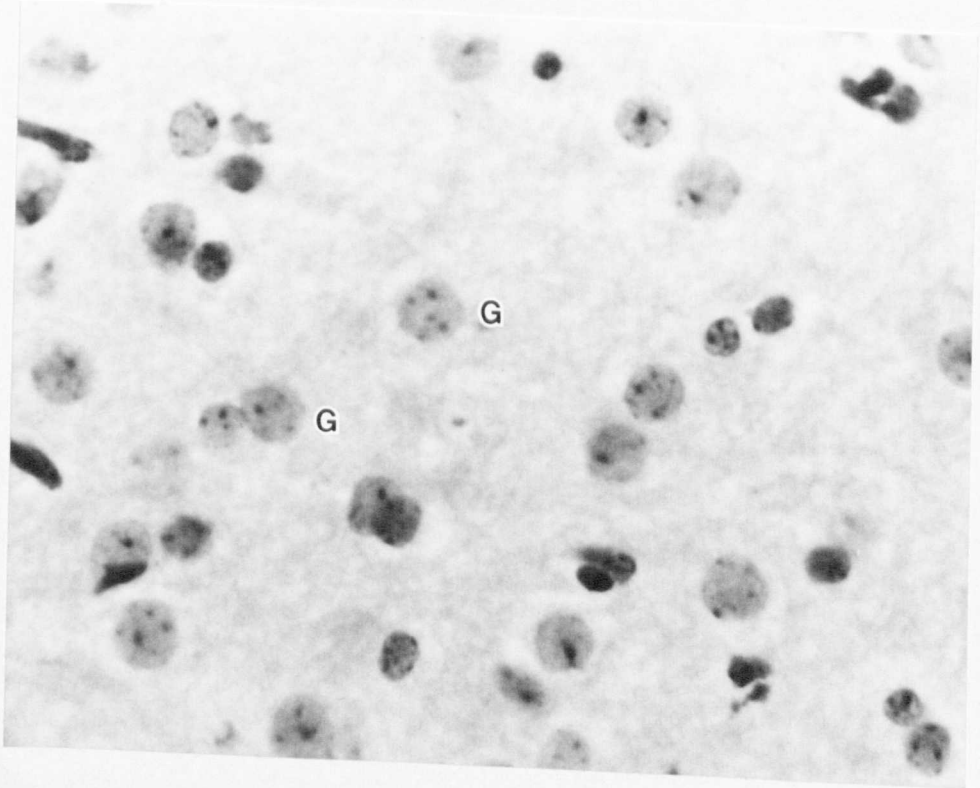


Figure 41:



Figure 42:

**Figure 43:**     Internal aspect of intact <u>Toxoplasma</u> tissue
cyst showing relatively homogenous
deposition of <u>Toxoplasma</u> antigen within the
matrix (M) surrounding the cystozoites (C)
Relatively little antigen is associated with
the cystozoites.
(Colloidal gold Mag X42000).

**Figure 44:**     Perifery of intact <u>Toxoplasma</u> tissue cyst
showing heavy deposition of <u>Toxoplasma</u>
immediately interior to the parasitophorous
vacuole membrane (P) the structure which
delimits the cystozoites (C). Relatively
little antigen is directly associated with
the cystozoites. (Colloidal gold MagX42000).
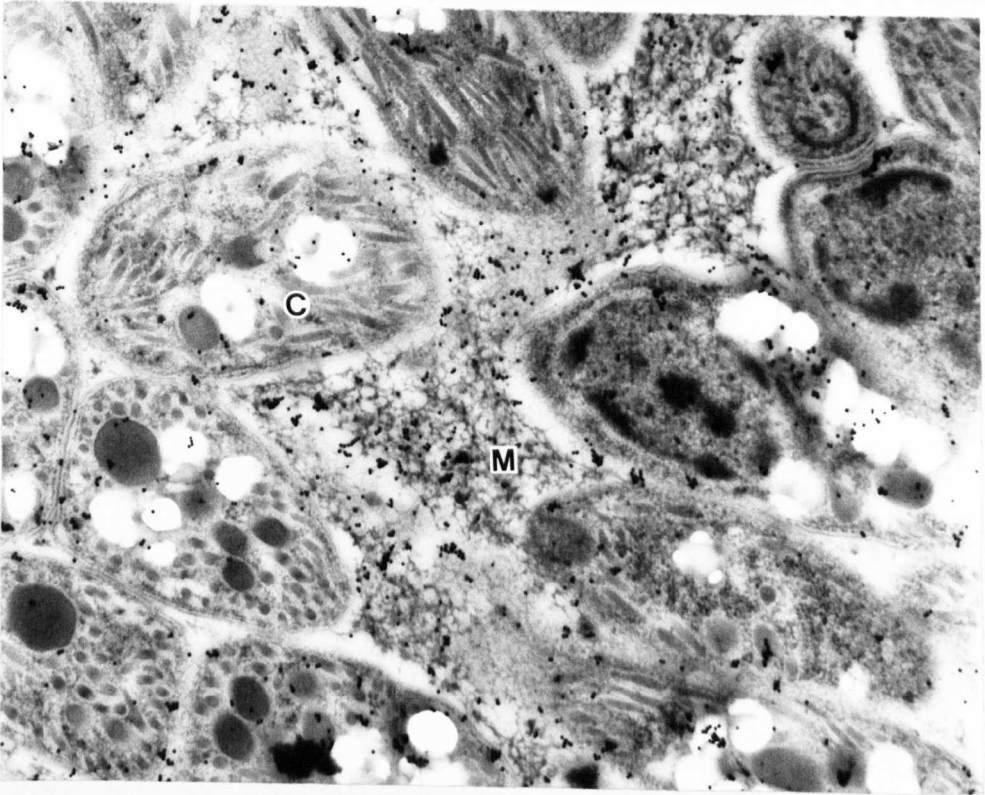
Figure 43:
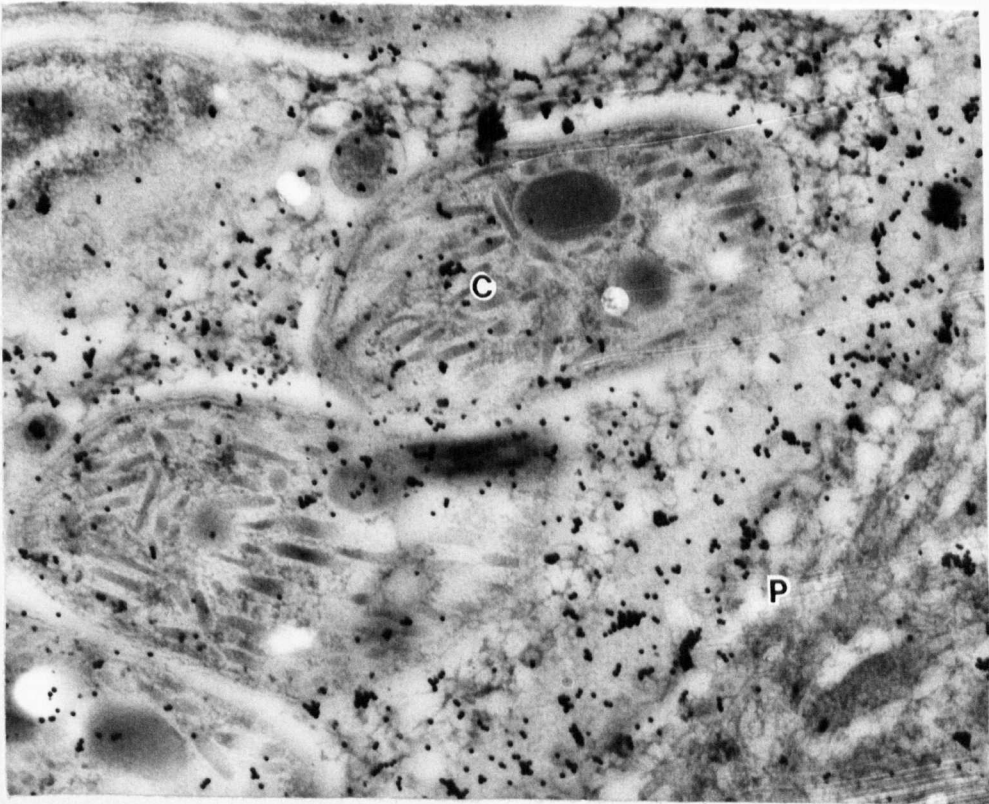


Figure 44:

**Figure 45:**      Periphery of an intact <u>Toxoplasma</u> tissue

cyst showing features similar to Figure

44 (Colloidal gold Mag. x42000)

**Figure 46:**      Internal aspect of intact <u>Toxoplasma</u>

tissue cyst showing features similar to

Figure 43.

(Colloidal gold Mag X42000)
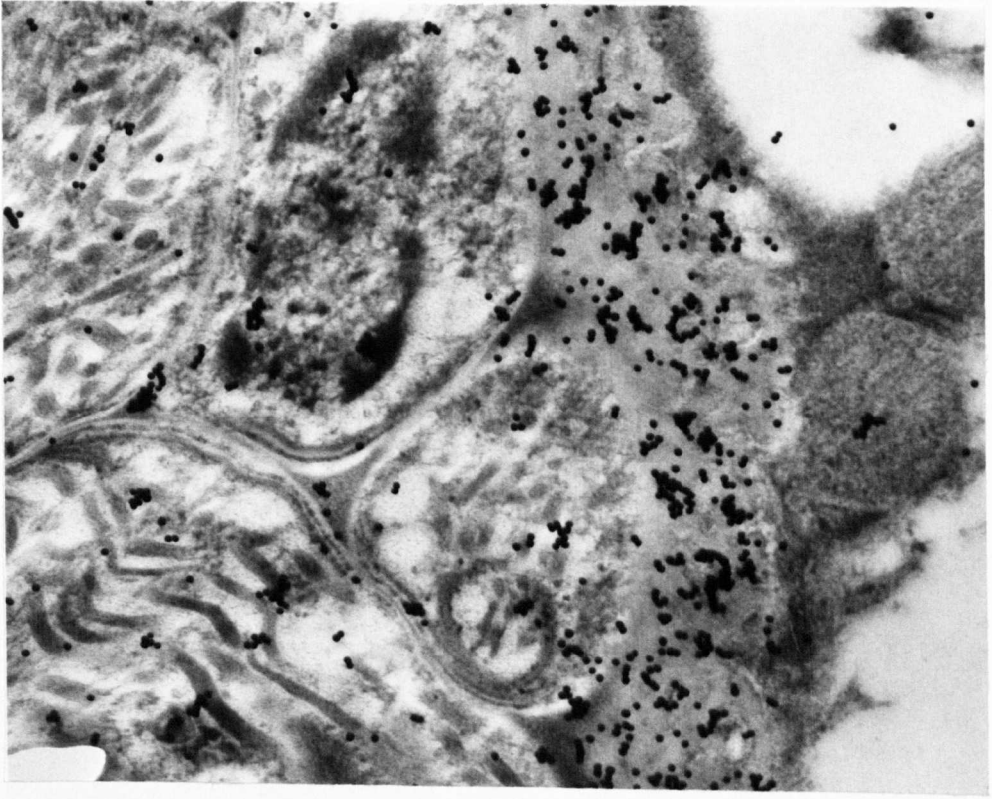
Figure 45:
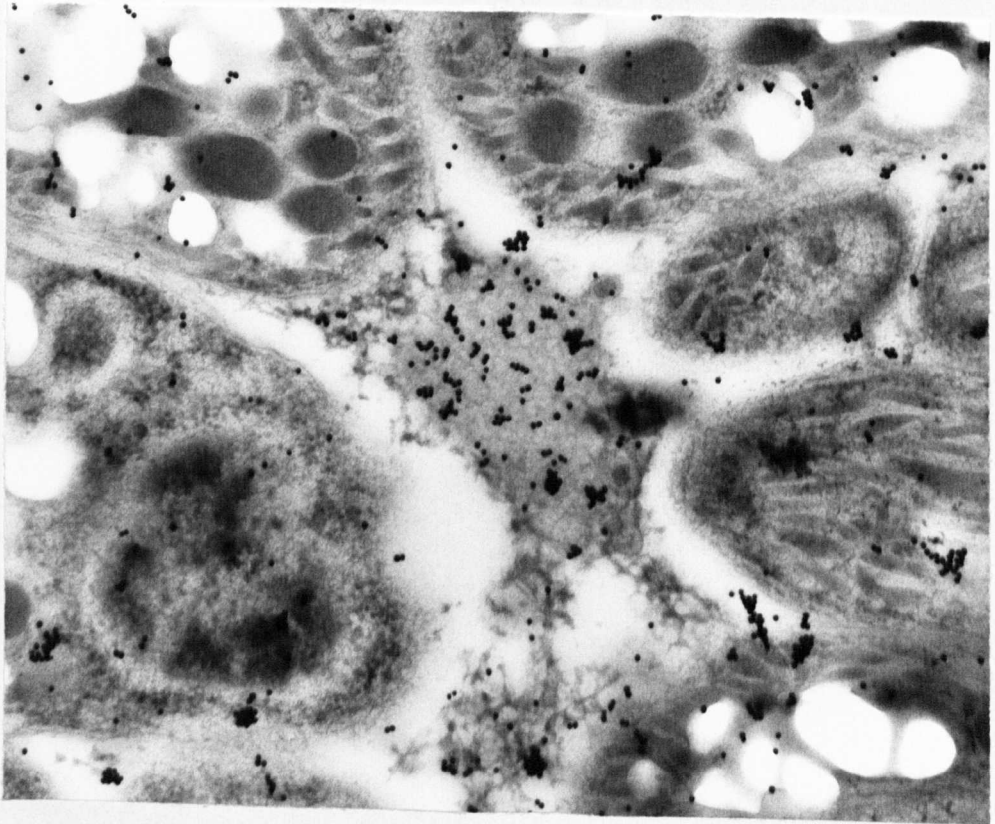


Figure 46:

Figure 47:    Internal aspect of an intact <u>Toxoplasma</u>

              tissue cyst similar to Figure 46.

              (Colloidal gold Mag. X50000).

Figure 48:    Periphery of an intact Toxoplasma tissue

              cyst showing deposition of <u>Toxoplasma</u>

              antigen within the matrix (M). In some

              instances there is no <u>Toxoplasma</u> antigen

              directly associated with the cystozoite (C).

              (Colloidal gold Mag. X42000).
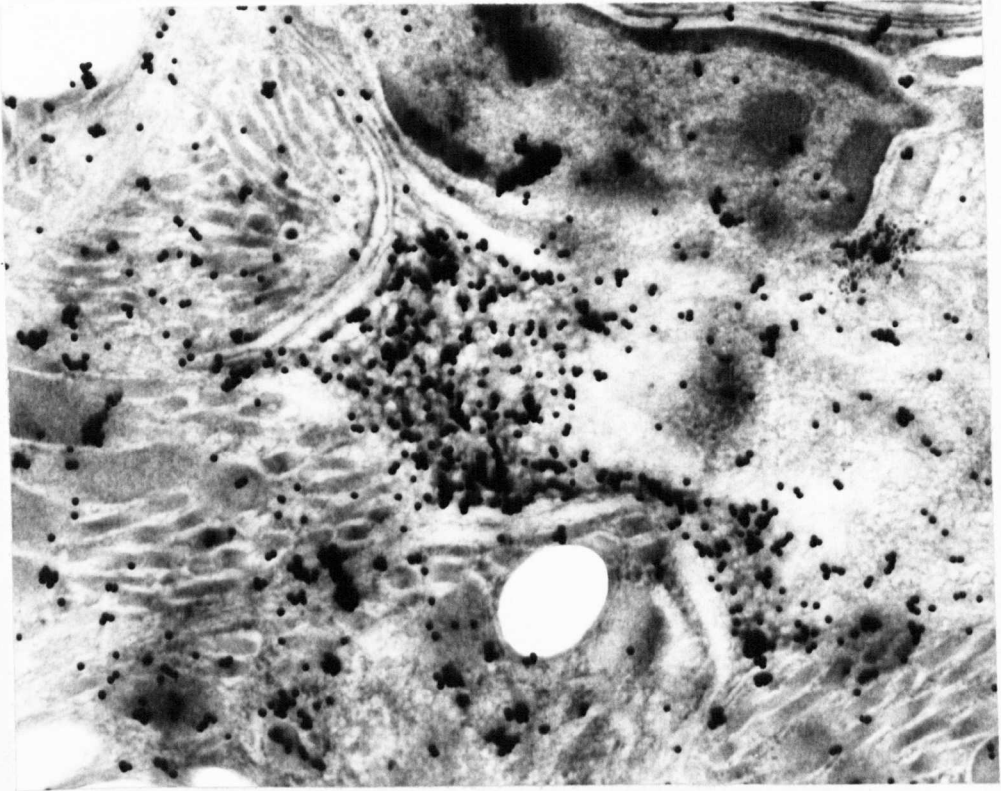
Figure 47:
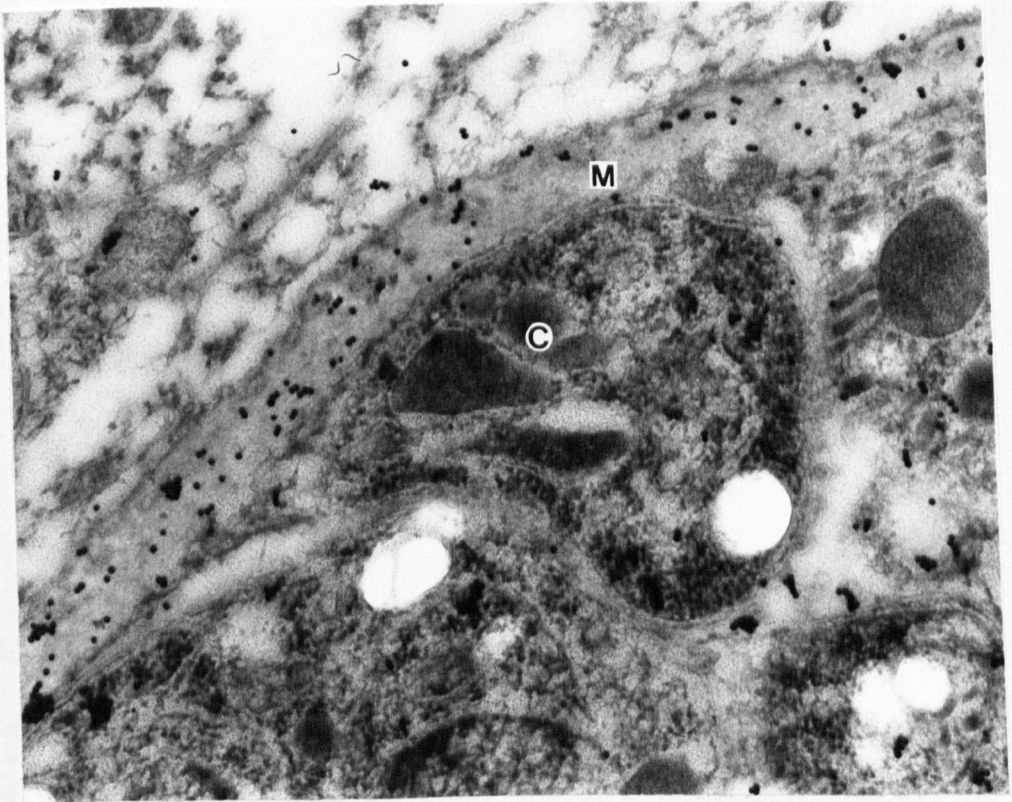


Figure 48:

**Figure 49:**    Internal aspect of intact <u>Toxoplasma</u> tissue

cyst similar to area in Figure 48.

Anti-<u>Toxoplasma</u> IgG has been omitted from

the immunocytochemical staining protocol; no

<u>Toxoplasma</u> antigen is discernable

(Mag. X42000)

**Figure 50:**    Peripheral aspect of an intact <u>Toxoplasma</u>

tissue cyst similar to the area shown in

Figure 46. Anti-<u>Toxoplasma</u> IgG has been

omitted from the immunocytochemical protocol

(Colloidal gold Mag X42000).

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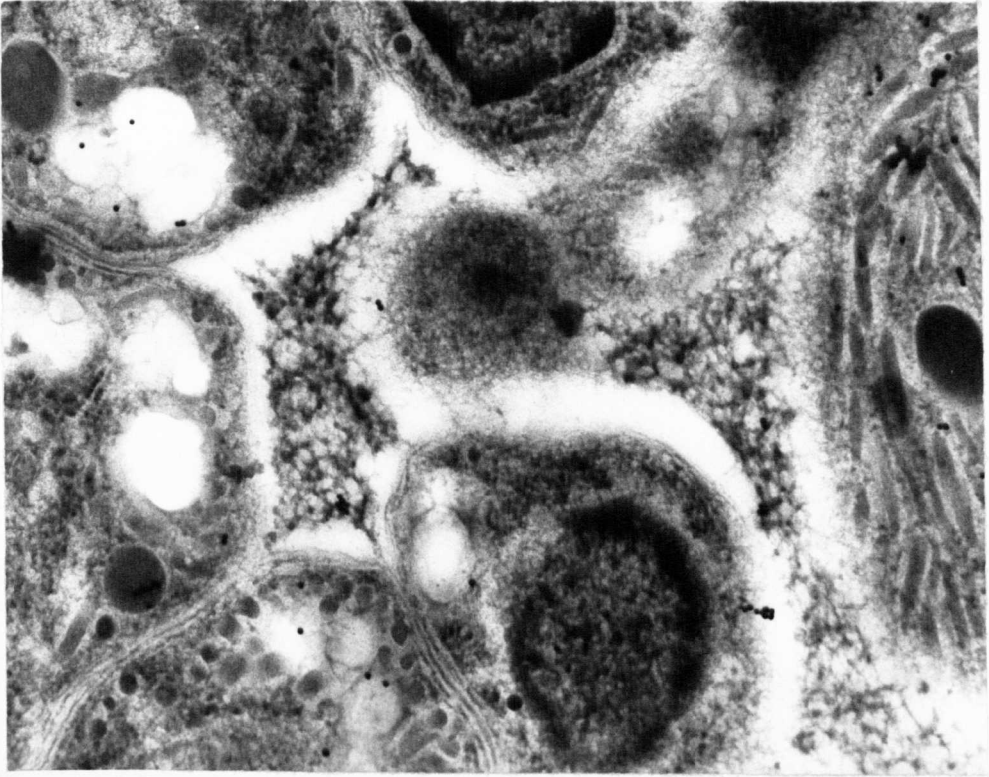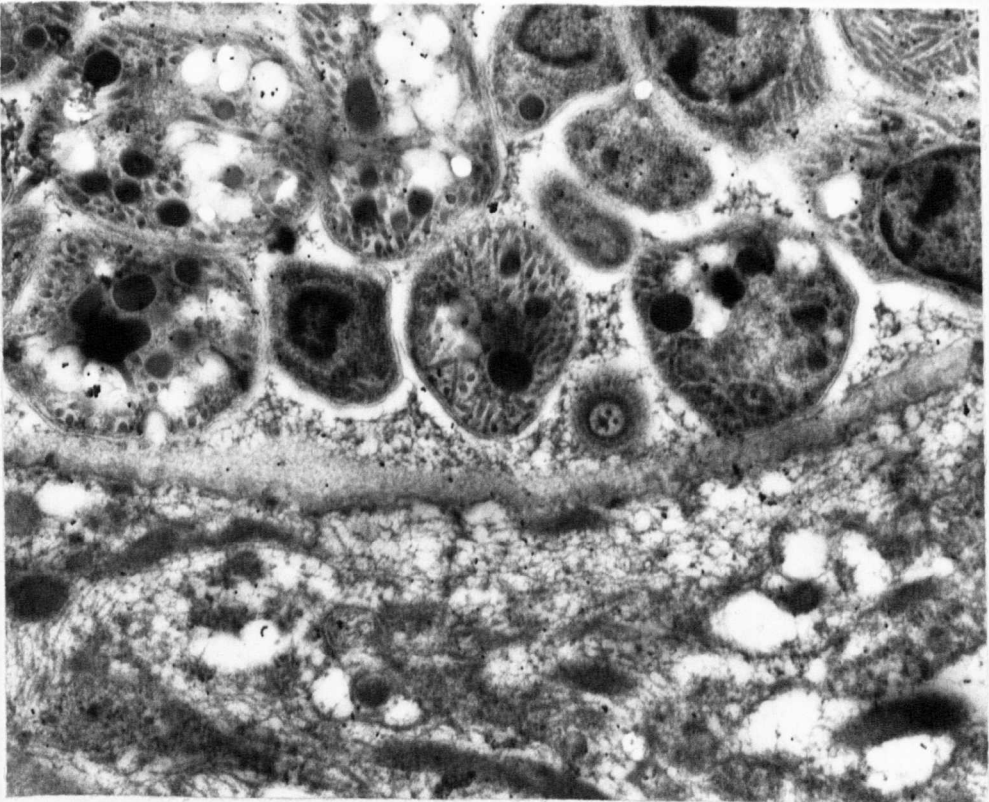


Figure 49:



Figure 50:

Figure 51:    Periphery of two adjacent intact Toxoplama
              tissue cysts showing heavy deposition of
              Toxoplasma antigen immediately interior to
              the parasitophorous vacuole membrane
              (Colloidal gold Mag X42000).

Figure 52:    Periphery of adjacent Toxoplasma tissue
              cysts shown in figure 51. Anti-Toxoplasma
              IgG has been omitted from the
              immunocytochemical staining protocol.
              (Colloidal gold Mag X42000).
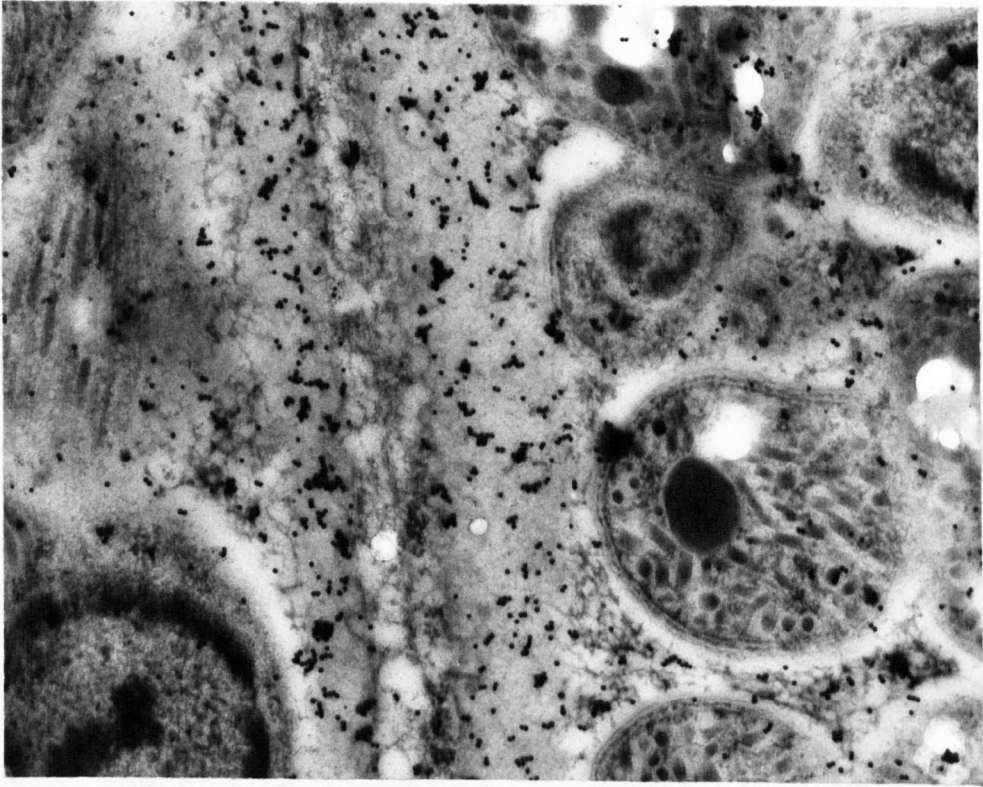
Figure 51:
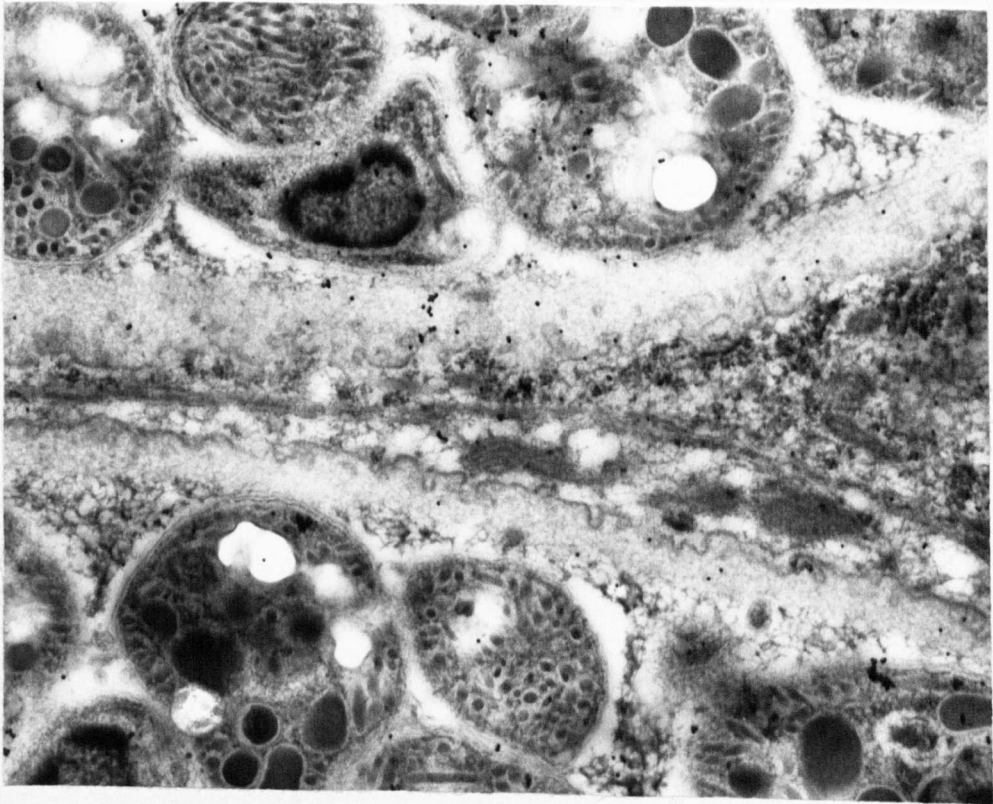


Figure 52:

## 3.4.2    QUANTITATIVE ASPECTS

Findings from the electron micrographs showing deposition
of immunogold particles (IP) were quantified  It was
considered that merely scanning the micrographs by eye
might be over-subjective. Thus certain histometric
measurements were taken using the DIGIT (Agar Aids) image
analysis software package with the BBC Master Micro and BIT
PAD 2 Digitizer Tablet.
The following parameters were measured.


1. Total area of micrograph.
2. Total area occupied by cystozoites.
3. Total immunogold particles in cystozoites.
4. Total immunogold particles in matrix.
5. Total area of micrograph
6. Total area occupied by cystozoites.
7. Total area outside parasitophorous vacuole
8. Total immunogold particles in cystozoites
9. Total immunogold particles in matrix.
10. Total immunogold particles outside parasitophorous
vacuole


Data in 1,2,3,4, relate to the area in the "centre" of
tissue cysts.*

Data in 5,6,7,8,9,10 relate to the area at the "periphery
of the tissue cysts**

* From 13 tissue cyst micrographs

** From 20 tissue cyst micrographs

The data acquired was then transferred to the Leicester
University VAX cluster mainframe computer. Data was then
analysed using appropriate statistical tests (see below)

using the SPSS-X statistical software package (SPSS Inc 1986)

Table 1 shows that the data was slightly skewed and kurtotic. Thus it was determined to use non-parametric statistical analysis to test the data.

---

**TABLE 1**

|  | SKEWNESS | KURTOSIS |
|---|---|---|
| CYSTOZOITES | -0.257 | -1.488 |
| MATRIX | +0.328 | +0.229 |
| "HOST" | +2.577 | +6.527 |

Table 2 shows the results of a Friedman analysis of variance. This showed that there was a highly significant statistical difference between the variables.

---

**TABLE 2**

|  | RANK | $x^2$ | P |
|---|---|---|---|
| CYSTOZOITES | 1.85 | 18.900 | =0.0001 |
| MATRIX | 2.75 |  |  |
| "HOST" | 1.40 |  |  |

Table 3a shows the median and range values for each variable

Table 3b shows the result of a series of Wilcoxon's signed ranked tests

**TABLE 3A**

|  | Median | Range |
|---|---|---|
| Cystozoites | 39.448 | 3.333-63.714 |
| Matrix | 99.258 | 7.778-245.238 |
| "Host" | 20.556 | 3.478-146.923 |

**TABLE 3B**

|  | Z | P |
|---|---|---|
| Cytozoites V Matrix | -3.7706 | =0.001 |
| Matrix V "Host" | -3.5839 | =0.001 |
| Cystozoites V "Host" | -1.5680 | =0.1169 |

Findings from Table 3a and 3b allowed the following

conclusions to be drawn:.

1. The matrix contained a greater number of immunogold

particles than the cystozoites.

2. The matrix contained a greater number of immunogold

particles than the "host" component.

3. There was no statistically significant differance in the

number of immunogold particles between the cystozoites and

the "host" component.

It could be argued that the differences were merely due to

different surface areas occupied by the various

components in the micrographs. In order to exclude this

explanation for the differences in immunogold deposition, a

series of Spearman's rank correlation coefficients were

computed. The results are shown in Table 4 and these

clearly indicate that the findings presented in Tables 2,

3a and 3b are independent of the surface area occupied by

the individual components in the micrographs.

## TABLE 4

---

|  | Coefficient | Z tested P |
|---|---|---|
| Cystozoites v Cystozoite area | +0.3684 | =0.110 |
| Matrix v Matrix area | -0.1440 | 0.545 |
| "Host" v "Host area" | +0.0211 | 0.930 |

---

## 3.5    VASCULAR STUDIES

## 3.5.1    INTRODUCTION.

Inflammatory cell infiltrates which appear to induce

Toxoplasma-associated pathology in the brain and elsewhere,

are likely to be attracted to antigen from tissue cysts

(Section 3.4.) In order to gain access to the tissue, the

inflammatory cells must first pass through the small

vessels in the vicinity of the tissue cyst.

The purpose of this component of the study is to record the

findings of an ultrastructural study, the primary aim of

which was to examine the pathological changes associated

with such small vessels in the proximity of intact

Toxoplasma tissue cysts in the brains of mice with

congenital Toxoplasma infection (section 2.5), which was

clinically inapparent. As an extrapolation of this

morphological study, it was hoped to provide a possible

explanation as to how intracystic parasites might be

released from the intact tissue cyst into the brain, to

initiate the neuropathological lesions associated with

progressive congenital toxoplasmosis. The study used the

tissue samples prepared for electron microscopic

examination as described in section 2.12. and fresh frozen

tissue as described in section 2.6.

A subsequent study extended these purely morphological aspects and involved the selective immunolabelling at the light microscope level, of the mononuclear inflammatory cell infiltrate associated with the vascular lesions.

## 3.5.2. ELECTRON MICROSCOPY

A common feature of light microscopic examination of the brain was an intact tissue cyst of <u>Toxoplasma</u> which elicited no obvious inflammatory response in its immediate vicinity; however, in the same field there was invariably small vessel pathology (Figure 53).Ultrastructurally, this lesion took the form of a minimal perivascular inflammatory cell infiltrate comprising mainly lymphocytes, but also including macrophages, plasma cells and neutrophilic granulocytes (Figure 54). In some vessels, individual (Figure 55), or groups (Figure 56) of lymphocytes, were seen in intimate contact with the luminal surface of the endothelium of the vessel. Rarely, lymphocytes were observed in the process of penetrating the intercellular space between the endothelial cells, into the underlying basal lamina (Figure 57). The basal lamina of endothelial cells of affected vessels was often thickened; in such cases electron dense flocculent material was present (Figure 58). Occasionally, vacuolation in the region of the basal lamina was observed, a feature compatible with oedema (Figure 59).

Noteworthy was the presence in some sections of lymphocytes attached to the outer host-cell component of intact Toxoplasma tissue cysts. (Figure 60).

### 3.5.3.    IMMUNOCYTOCHEMISTRY

The CD5 marker for T (strong) and B (weak) cells, showed these cell types to be present in both, vessels with small numbers (Figure 62), and those with large numbers (Figure 61),of inflammatory cells. The CD8 marker for cytotoxic and suppressor T-cells, showed a similar pattern of staining, but the number of cells staining with this antibody was significantly reduced relative to that observed with the CD5 marker in its T-cell mode. (Figures 63 and 64); by inference therefore, it was possible to speculate that the predominant T-cell type in the perivascular inflammatory areas, was either the T helper or T inducer cell. However, no monoclonal antibody (CD4) was available for the demonstration of these T cell types in murine tissue. The CD19 marker for B cells, showed a similar pattern of staining as shown by the CD5 marker in its B cell mode. This monoclonal antibody provided  confirmation (by virtue of its more intense staining), that B-cells were present in both, areas where inflammatory cell numbers were low (Figure 65) and in those areas where inflammatory cells were present in greater number (Figure 66); however,

relatively, the B cell type of lymphocyte predominated in the former type of lesion.

The staining of macrophages was similar to that for T cells, as determined by results observed from CD14 staining (Figure 67).

With all four monoclonal antibodies, staining of endothelial cells was noted.

Figure 53:     Toxoplasma tissue cyst in congenitally
               infected mouse brain. No obvious direct
               inflammatory response is seen associated
               with the cyst, but small blood vessels
               in close proximity show marked perivascular
               cuffing of inflammatory cells.
               (H and E Mag X400)


Figure 54:     Electron micrograph of perivascular
               inflammatory infiltrate consisting of
               lymphocytes (L), and macrophages (M).
               (Mag X3100).
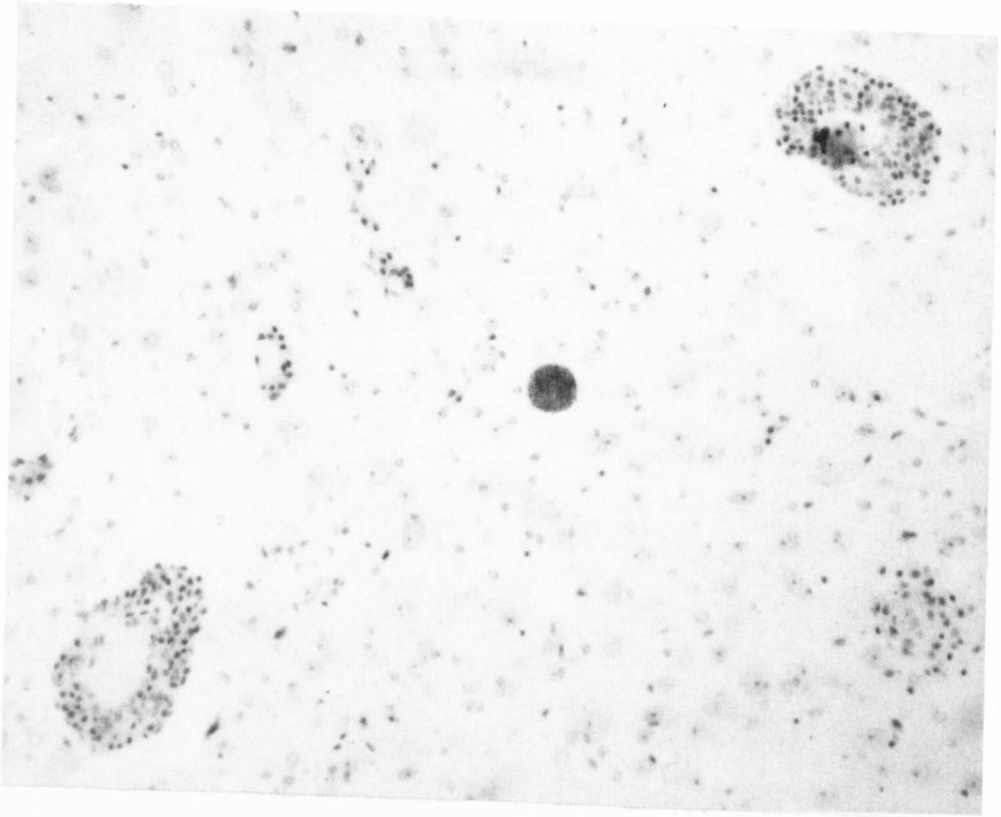
Figure 53:
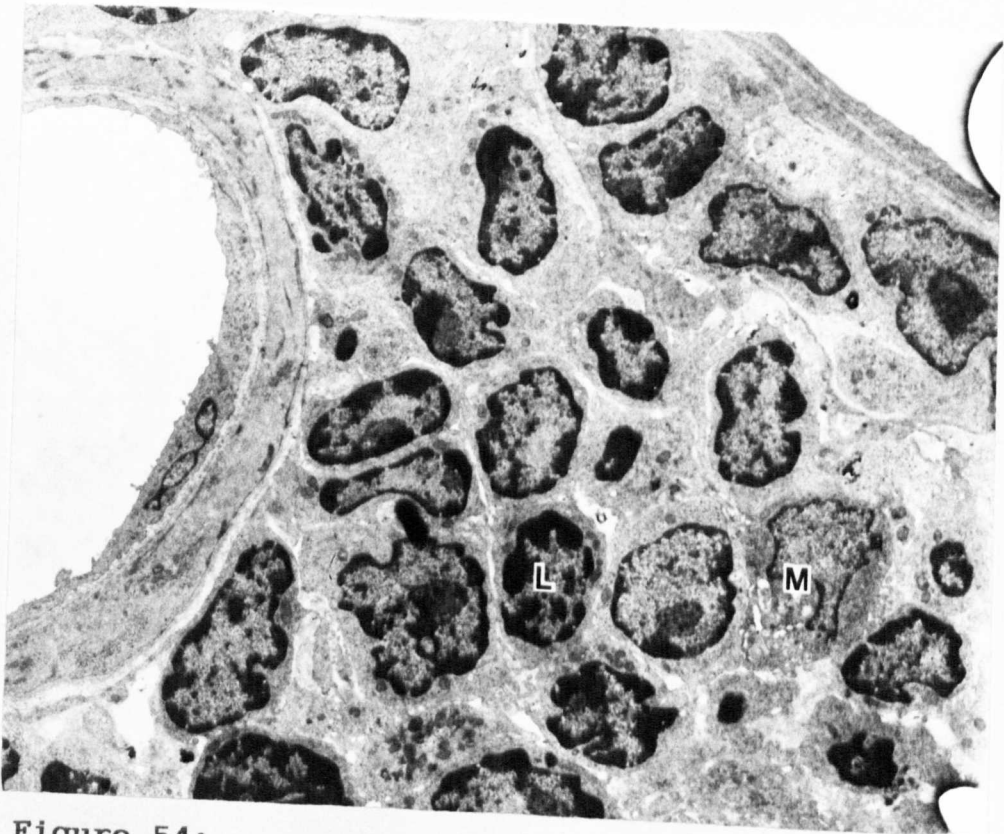


Figure 54:

**Figure 55:**     Capillary lumen showing endothelial cell (E)
and flocculent basal lamina material (BL).
On the luminal surface is a lymphocyte in
close apposition to the endothelial cell.
(Mag X10000).

**Figure 56:**     Capillary lumen showing endothelial cells
(E) with three lymphocytes in close
apposition on the endothelial cell luminal
surface.
(Mag X8500).

84
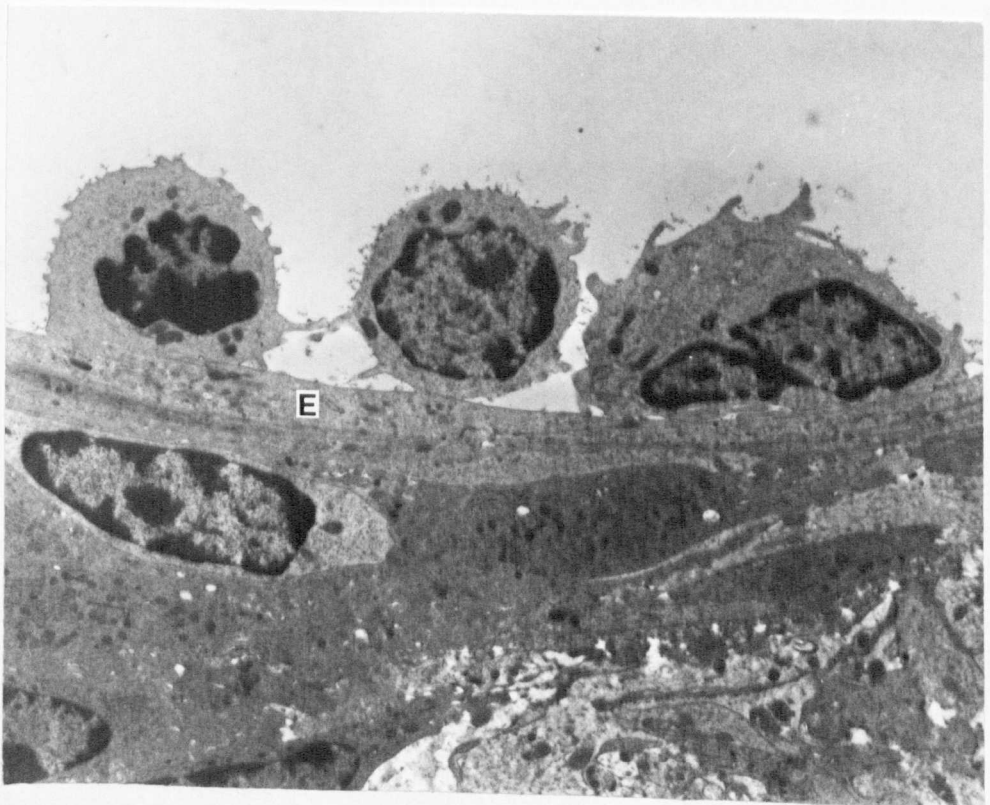


Figure 55:



Figure 56:

Figure 57:   Two lymphocytes (L) breaching the
             intercellular space between capillary
             endothelial cells and almost traversing
             the basal lamina (BL)
             (Mag X12100).

Figure 58:   Small capillary showing perivascular
             inflammatory cell infiltrate consisting
             of lymphocytes (L), neutrophils (N),
             plasma cells (P) and macrophages (M).
             Note the flocculant electron dense basal
             lamina material beneath the endothelial
             cells (F)
             (Mag X4000).
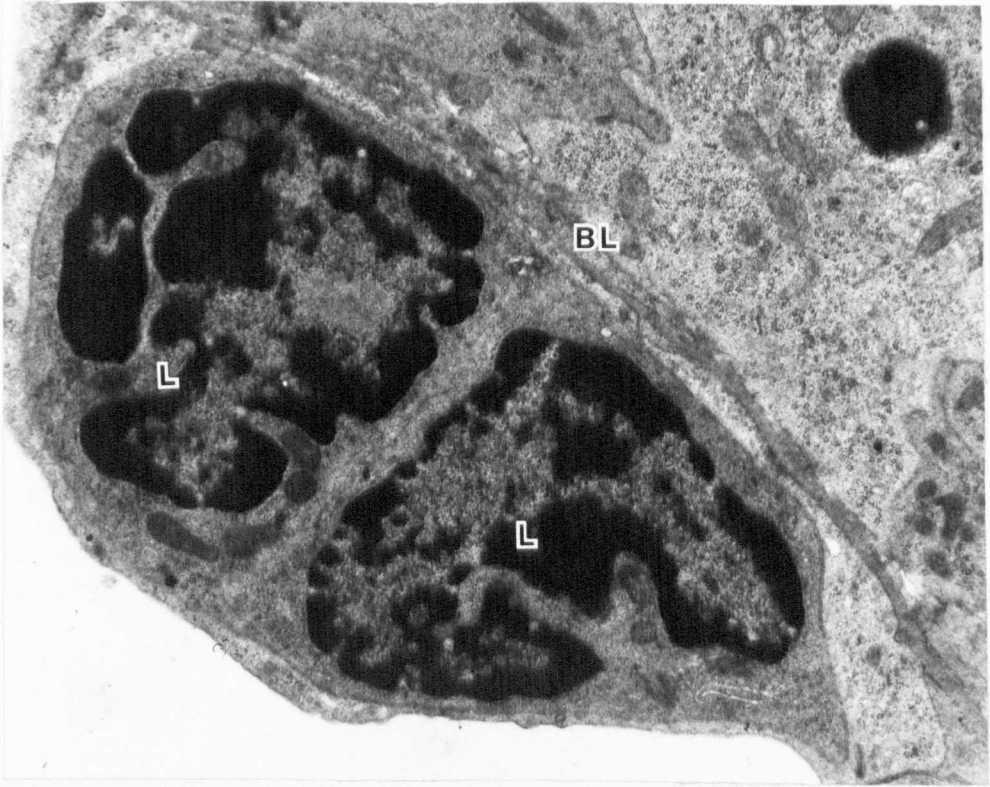
Figure 57:
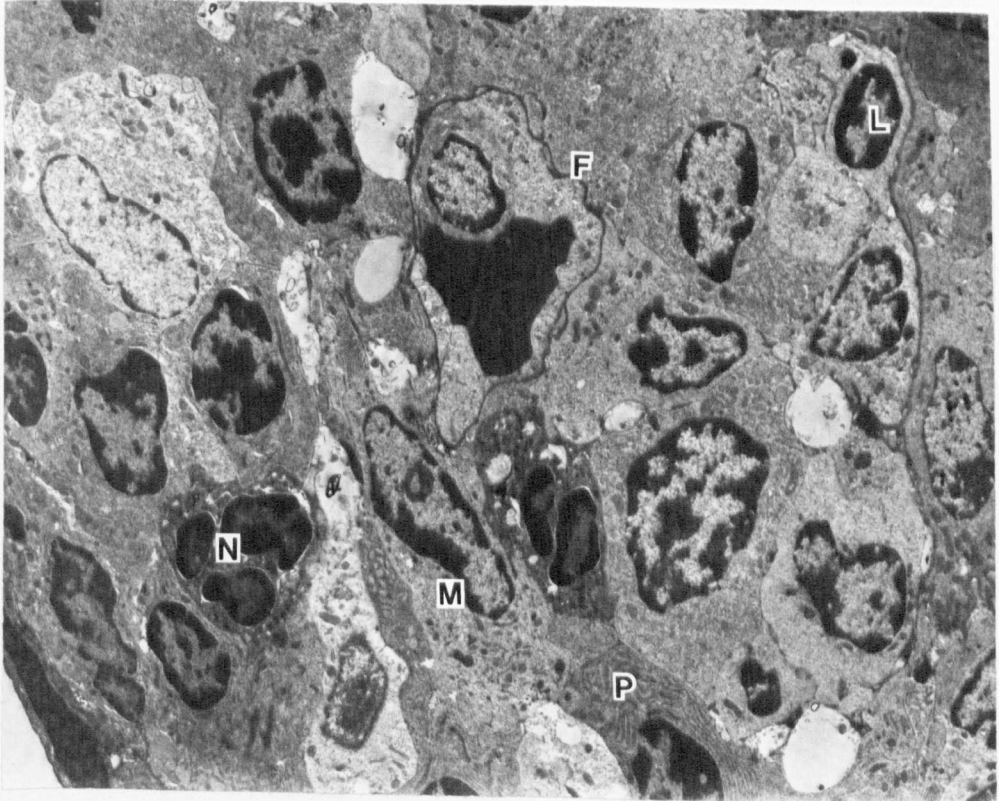


Figure 58:

**Figure 59:**   Small capillary showing marked extracellular vacuolation in the sub basal lamina region. (Mag X5300).

**Figure 60:**   Intact Toxoplasma tissue cyst in the brain of a congenitally infected mouse showing two lymphocytes (L) lying in close contact with the outer host cell component of the cyst. (Mag X6000).
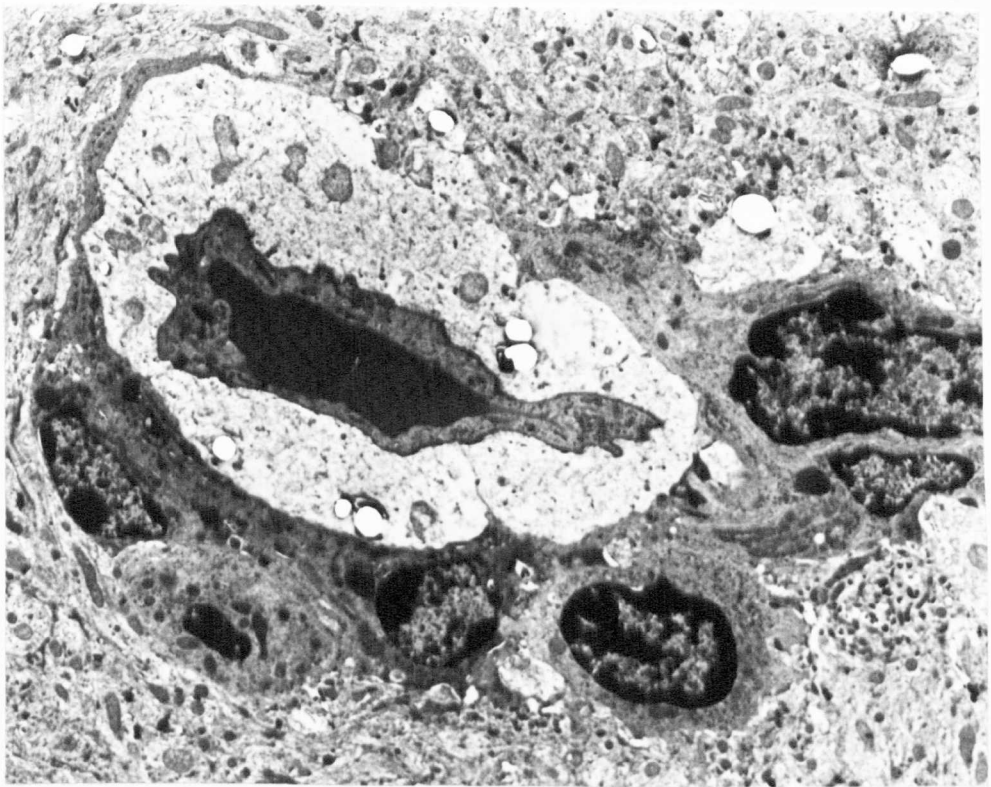
Figure 59:
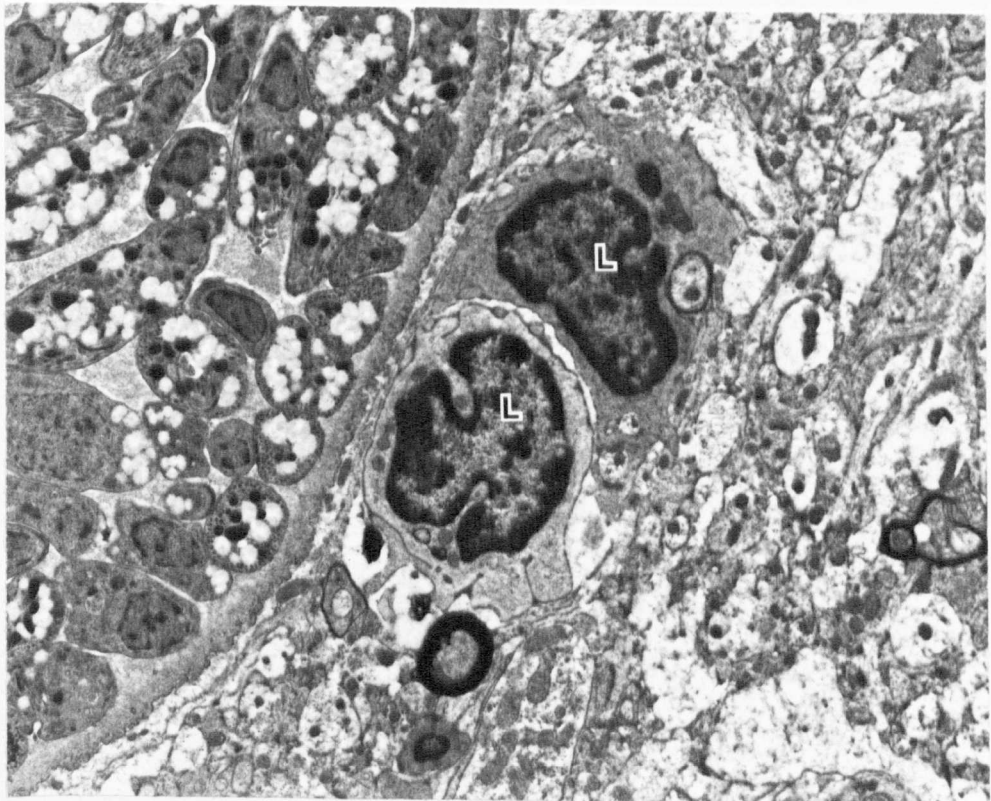


Figure 60:

**Figure 61:**   Perivascular inflammatory infiltrate

consisting of numerous T lymphocytes

exhibiting CD5 antigen.

PAP/Haemalum Mag X400.

**Figure 62:**   Perivascular inflammatory cell infiltrate

consisting of small numbers of T lymphocytes

exhibiting CD5 antigen.

PAP/Haemalum Mag X400.
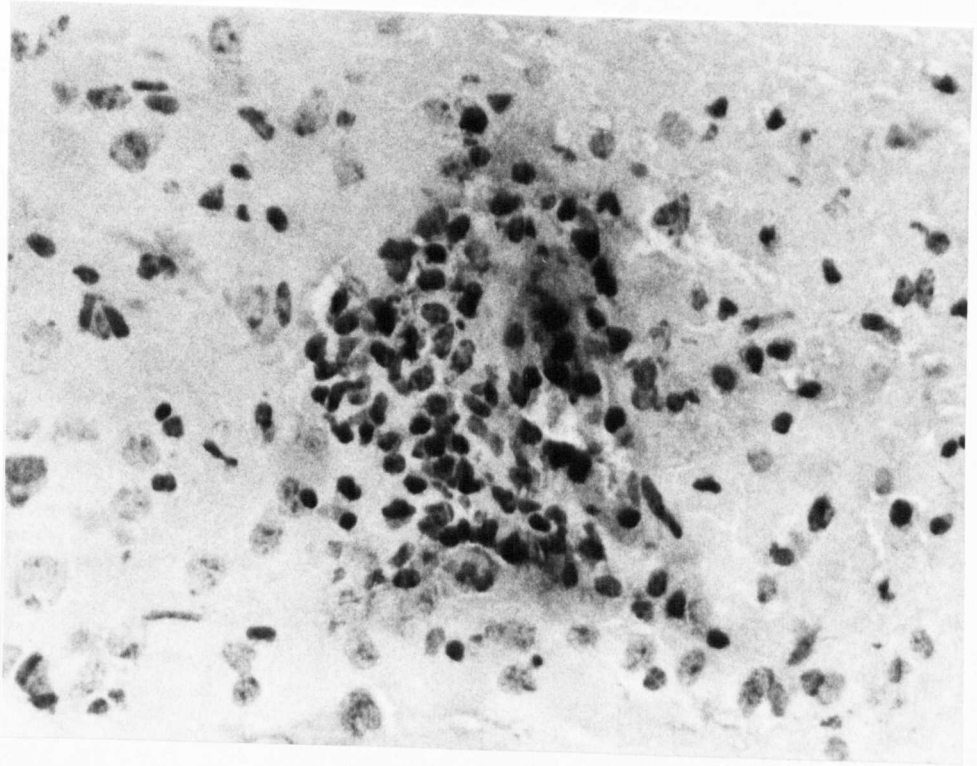
Figure 61:
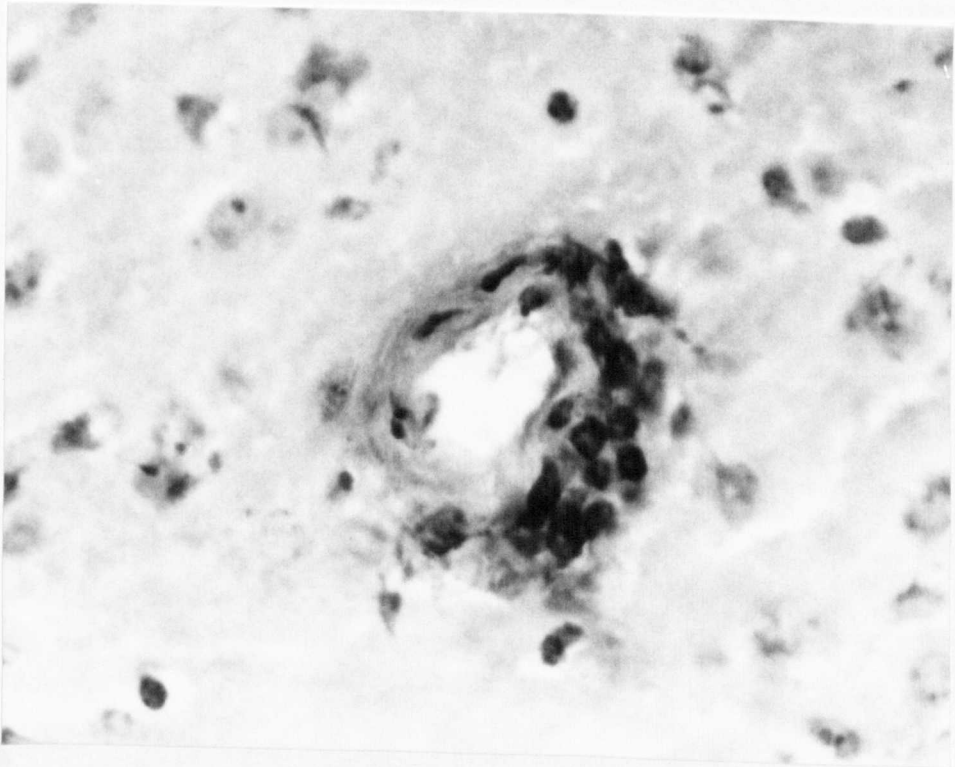


Figure 62:

Figure 63:     Small blood capillary with perivascular
               "cuff" of T lymphocytes exhibiting CD8
               antigen.
               PAP/Haemalum Mag X400.

Figure 64:     Small blood vessel with numerous T
               lymphocytes exhibiting CD8 antigen
               expression.
               PAP/Haemalum Mag X400.
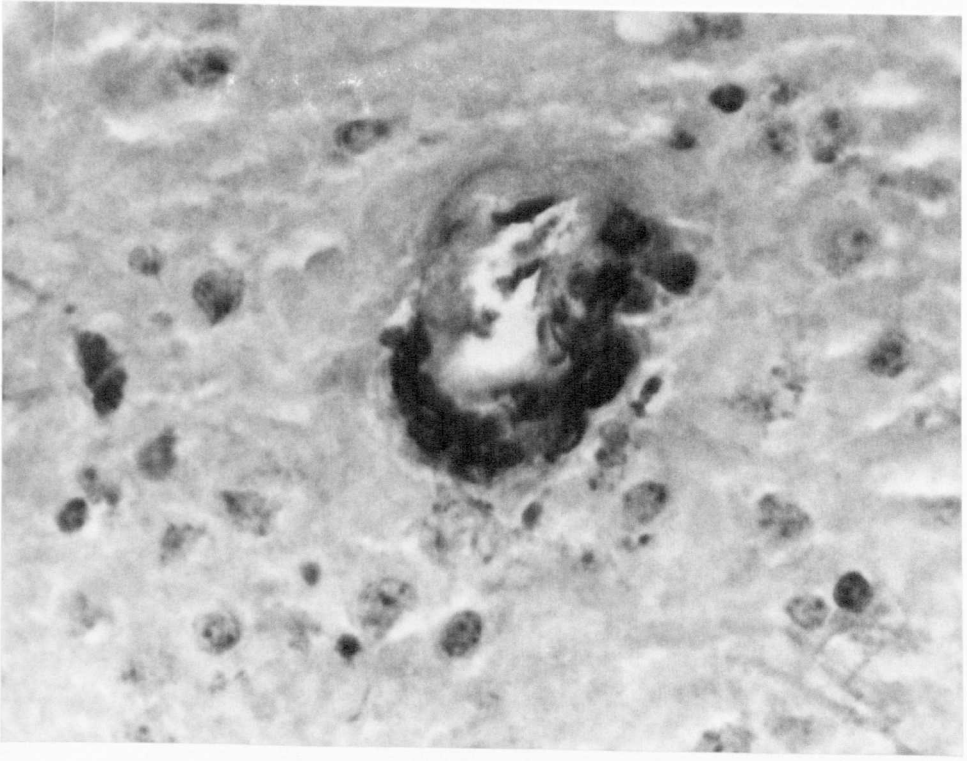
Figure 63:
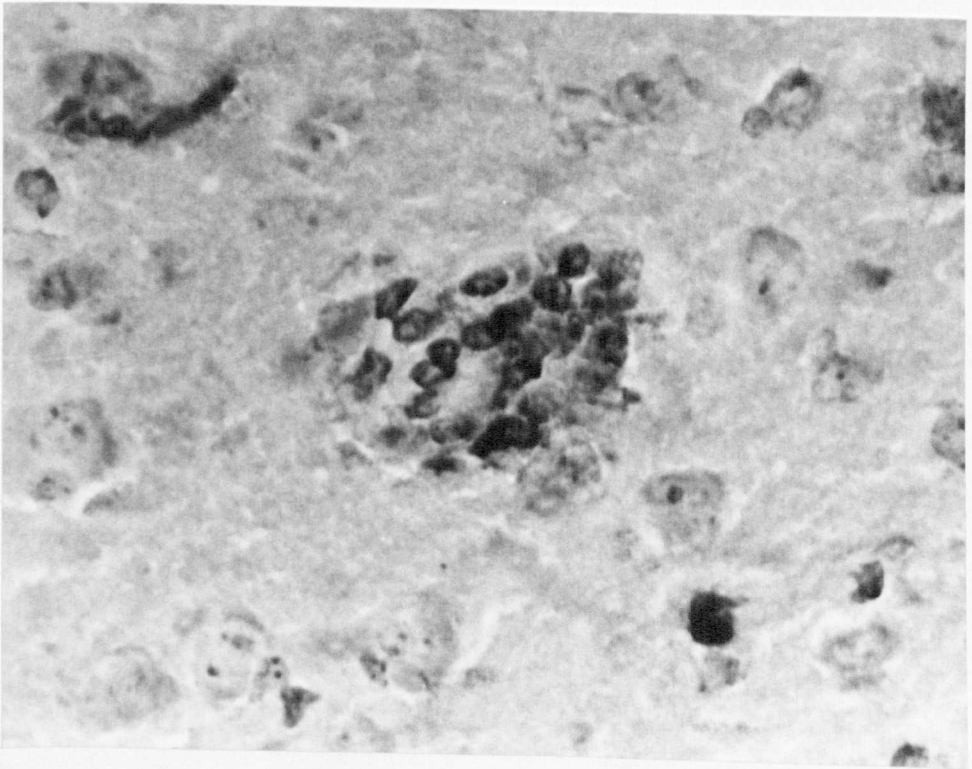


Figure 64:

**Figure 65:**    Perivascular inflammatory cell infiltrate consisting of numerous B lymphocytes exhibiting CD19 antigen.
PAP/Haemalum Mag X400.

**Figure 66:**    Perivascular inflammatory cell infiltrate consisting of small numbers of B lymphocytes exhibiting CD19 antigen.
PAP/Haemalum Mag X400.
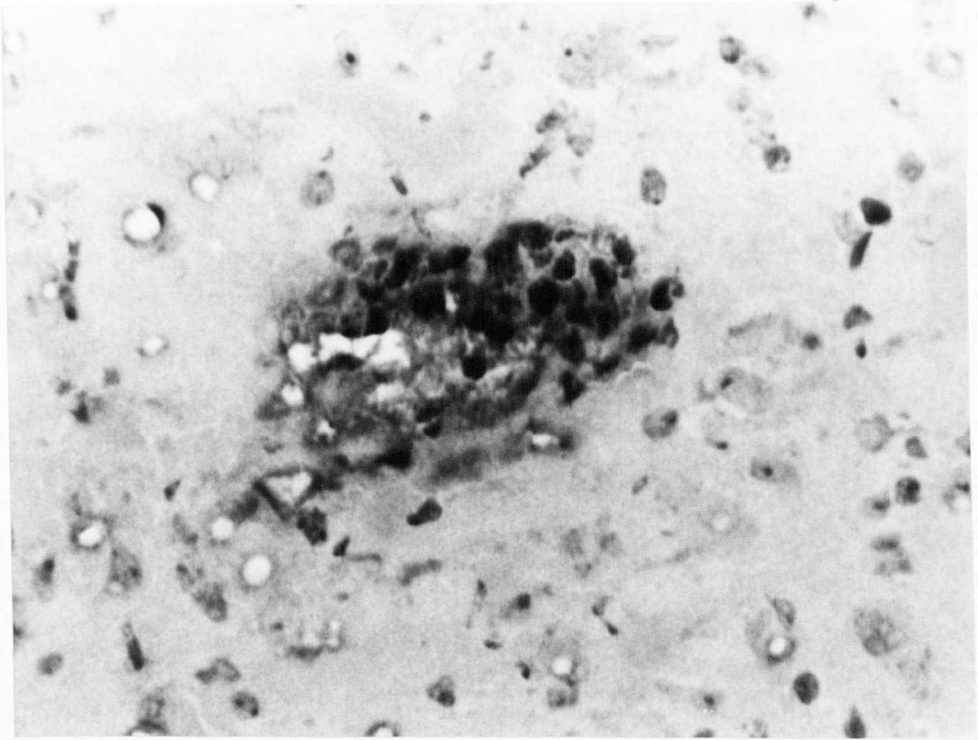
Figure 65:
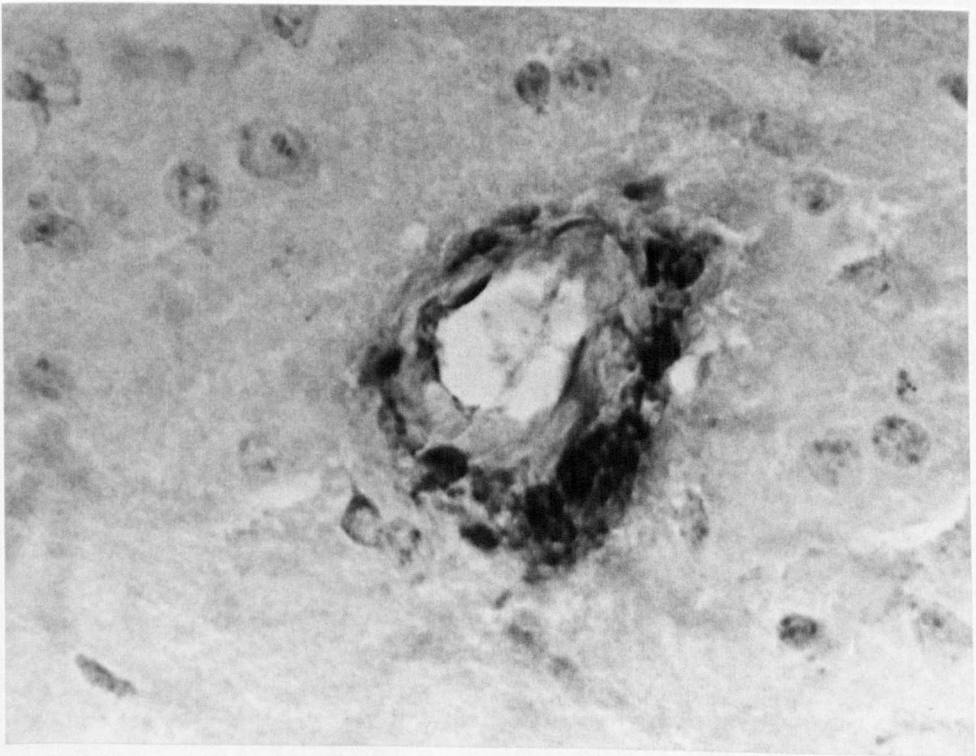


Figure 66:

Figure 67:      Perivascular inflammatory cell infiltrate with small numbers of macrophages exhibiting CD14 antigen ( > ). PAP/Haemalum Mag X400.
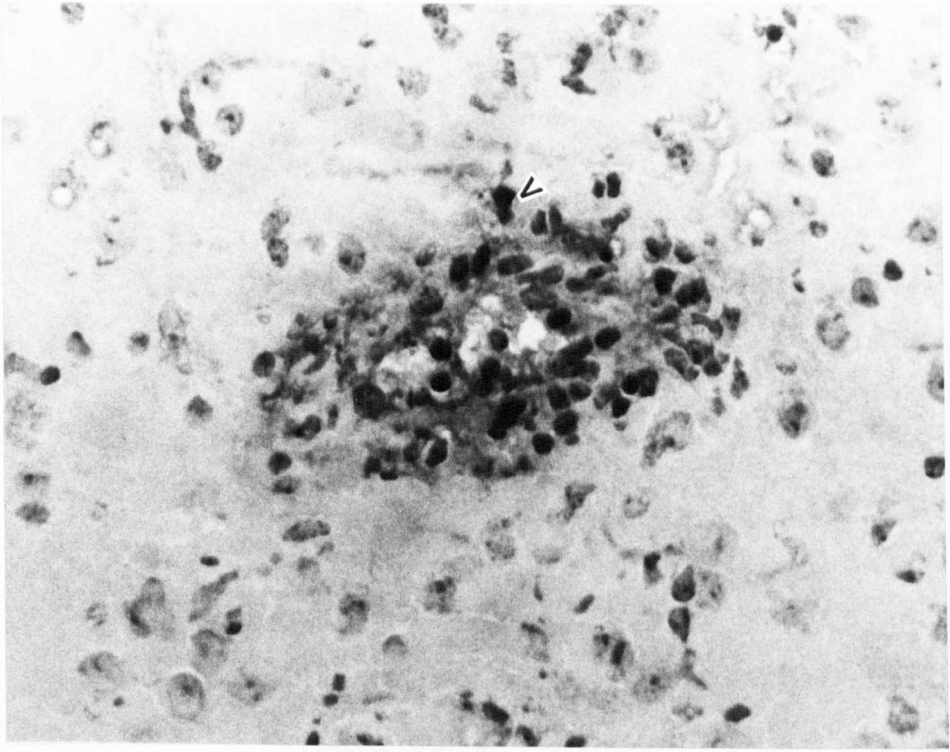
Figure 67:

CHAPTER 4

DISCUSSION

**4.1        OBSERVATIONS ON THE NATURE OF THE CYST "WALL"**

**OF TOXOPLASMA TISSUE CYSTS**

Neurological sequelae of Toxoplasma infection are commonly associated with congenital toxoplasmosis (Remington et al. 1983) and may even be present in apparently subclinical infections acquired by this route (Wilson et al. 1980). Investigation of any underlying neuropathology in such cases is hampered by an inevitable paucity of human cerebral tissue. The brains of the murine model of the disease used in this study, which results from exposure of a dam to a primary Toxoplasma infection during pregnancy, showed histopathological features (Graham et al. 1984) which appear similar to those seen in human congenital toxoplasmosis. This mouse model, therefore, offered the opportunity for the investigation of the inter-relationship between the parasite, present in chronic toxoplasmosis as tissue cysts, and the brain of the host.

The initial part of the present study involved the use of conventional light and electron microscopy in order to examine the host-parasite relationship in the brain of mice with subclinical toxoplasmosis.

Tissue cysts of Toxoplasma develop intracellularly; the contained cystozoites multiply by endodyogeny (Goldman et al, 1958) within the parasitophorous vacoule. It has been

suggested that the tissue cyst may become extracellular as a result of "rupture" of the host cell membrane (Van der Zypen, 1966). However, it remains uncertain whether intact tissue cysts ever leave the host cell at any stage of their existence (Dubey, 1977), If they do so, it is necessary to explain why large tissue cysts, having no obvious association with their original host cell, appear to provoke no inflammatory response, at least in their immediate vicinity.

One explanation would be that the tissue cyst "wall" is composed, at least in its outermost part, of host-derived constituents. Thus, it has been proposed from morphological studies in mouse brain that part of the wall may be derived from host cell endoplasmic reticulum (Wanko et al. 1962), or alternatively, from the host cell membranes (Uga et al. 1980). The finding of weak PAS staining and argyrophilia has been reported previously (Frenkel and Friedlander, 1951). The lack of staining with methenamine silver would tend to indicate that the "wall" contains no glycogen or other polysaccharide components. The argyrophilic nature of the "wall" provides circumstantial evidence that it may contain elements derived from the neuronal cytoskeleton, since in the brain, neurofibrillae are stained with reduced silver salts (Bray and Gilbert, 1981; Wuerker and Kirkpatrick, 1972).

The impregnation of the "wall" with protargol silver further suggests that it has incorporated into it components derived from the neuronal cytoskeleton. Although the latter procedure has many modifications which may produce multiple degrees of selective staining (Zagan et al. 1970), the method used in this present study (Clark, 1973) is generally regarded as specific for neuronal elements within the nervous system (Gregory, 1980). It has been shown that this specific affinity of protargol silver is for polypeptide units of neurofilament protein (Gambetti et al, 1981), structures which together with neurotubules, comprise the neurofibillae of the neuronal cytoskeleton. The ultrastructural morphology of the "wall" confirmed the light microscope observations. The presence of synaptic terminals on the outermost host cell membrane suggest that the parasitised cell remains functional.

Parasites may leave the host cell simply by mechanical pressure resulting from continued expansion of the tissue cyst (Frenkel and Escajadillo, 1987). However, faint staining of the "wall" has been observed at the light microscope level after treatment of paraffin sections with anti-Toxoplasma antibody and peroxidase anti-peroxidase staining (Conley and Jenkins, 1981; Hay et al, 1986). This may represent parasitic antigen diffusing out of the tissue cyst (Werner et al, 1981). It is possible that such antigen may interfere with the integrity of the

neurofibrillar proteins, or alternatively, may disrupt synaptic transmission to the host cell membrane. Under these conditions the host cell may begin to break down; the release of contained cystozoites and other _Toxoplasma_ antigens, would provoke an inflammatory response. Under these circumstances parasites not destroyed would be free to invade other cells in their immediate vicinity. This may explain the commonly recorded observation in infected brain tissue of inflammatory lesions in the vicinity, but not apparently related to, intact tissue cysts.

## 4.2. ELECTRON MICROSCOPE AND IMMUNOCYTOCHEMICAL STUDY OF THE INTRACELLULAR LOCATION OF _TOXOPLASMA_ TISSUE CYSTS IN THE BRAIN

In congenital infections with the _Toxoplasma_, focal brain lesions can result from primary proliferation of endozoites within the immunologically naive foetus or newborn infant (Remington and Desmonts, 1983). However, recrudescence of latent chronic infection may also have a role in the progression of congenital toxoplasmosis; prospective studies have shown that children born with subclinical _Toxoplasma_ infections may develop neurological sequelae in later life (Wilson _et al_. 1980).

Characteristic of the latent chronic stage of a <u>Toxoplasma</u>
infection in brain are tissue cysts which contain
cystozoites. The role of the tissue cyst in the
pathogenesis of toxoplasmic encephalitis and inflammation
of other tissues, is unclear. Tissue cysts are often
considered as dormant or slowly metabolizing stages in the
natural history of the parasite while it is within an
intermediate host (Frenkel and Escajadillo, 1987).

Central to an appreciation of the part played by tissue
cysts in the pathogenesis of postnatal congenital
toxoplasmic encephalitis, is the understanding of the host-
parasite relationship within the brains of individuals with
such an infection, and more specifically the nature of the
interface between the host neuropil and the cystozoite,
that is the so-called "wall" of the <u>Toxoplasma</u> tissue cyst.

It has been discussed above in Section 4.1 that the mature
intact tissue cyst appears to remain within its host cell,
which on morphological grounds has been identified as the
neurone. Transmission electron microscopy was used to
examine, at a specific time post-parturition, the
morphology of the exterior portion of intact tissue cysts
in the brain from mice with congenital toxoplasmosis.
Furthermore, immunohistochemical staining at the light
microscope level was utilised in order to determine

unequivocally the nature of the host cell component of these tissue cysts.

In all of the tissue cysts examined, in the study described in Section 3, the cystozoites were confined within an outwardly relatively smooth, but intensely folded, parasitophorous vacuole membrane. Exterior to this was a component comprising elements derived from the host cell. The microtubular element had a morphology and dimension comparable to neurotubules (Wuerker and Kirkpatrick, 1972; Bray and Gilbert, 1981) suggesting that they were of neuronal origin. Positive staining using neurofilament protein as a primary layer in the PAP method confirmed the morphological identification.

This observation has three major implications regarding the inter-relationship between the encysted _Toxoplasma_ organisms and the brain tissue of the host.

First, it has been suggested that intact tissue cysts may become extracellular as a result of rupture of the cell membranes (Van der Zypen, 1966). The results obtained from the present study indicate that, irrespective of the dimensions of the intact tissue cyst, it remains within the confines of the host cell. The presence of synaptic plates

on the outer host cell plasma membrane attest further (see Section 3) to the viability of the parasitised host cell (Peters et al, 1976).

Secondly, there is controversy as to the nature of the host cell component of the intact tissue cyst in the brain (Jones et al 1986). The findings from the present study reveal that intact tissue cysts are separated from the extra-cellular compartment by a layer of microtubules and neurofilaments, enclosed within the host cell membranes.

Thirdly, the identity of the host cell of Toxoplasma within the brain tissue has been variously described as mainly glial (Ghatak and Zimmerman, 1973; Powell et al. 1978), or mainly neuronal (Ferguson and Hutchison, 1987 a,b.). These conclusions were based on appraisal of ultrastructural features of remaining host cell component from the brains of humans, and from mice which had been experimentally infected in adulthood, respectively. Although the ultrastructural features observed in this present study were in agreement with those of Ferguson and Hutchison (1987), the immunocytochemical demonstration of neurofilament protein in the host cell layer of the intact tissue cyst "wall" provided unequivocal evidence that in the murine model of congenital toxoplasmosis used in this study, and at the stage of histological examination, all the tissue cysts observed were confined only within intact

functional neurones. Preferential parasitism of the neurone, a cell with a considerable life span, would be of considerable value for the survival of the parasite within the brain of its intermediate host. The limiting factor for the replication of the parasite in the brain, could be dependant mainly upon the elasticity of the host neurone and in particular the microtubular neurofilamentous layer, since the parasitophorous vacuole membrane was observed, even in very large tissue cysts, to be highly invaginated. This is in contradistinction to the microtubular components exterior to this membrane which were closely aligned in parallel.

## 4.3    IMMUNOELECTRON MICROSCOPY STUDY OF THE TOXOPLASMA TISSUE CYST IN BRAIN

Electron microscopy studies described in Sections 3 and 4 revealed that the lack of inflammation in the vicinity of the intact tissue cysts of Toxoplasma in the brain of mice with congenital toxoplasmosis resulted from the confinement of the tissue cysts within functioning neurones. Despite the lack of an inflammatory reaction, however, indirect immunofluorescence labelling of intact Toxoplasma tissue cysts has suggested that a soluble form of Toxoplasma antigen may diffuse from intact cysts into the brain of the host (Huldt, 1971).

Using the polyclonal anti-Toxoplasma antibody raised and prepared as in Section 2.14.2, and used previously by Hay et al (1985, 1986), electron microscopy revealed that Toxoplasma antigen was present primarily within the matrix of mature tissue cysts. Furthermore, the antigen was present in greatest concentration in the vicinity of the inner aspect of the parasitophorous vacuole membrane.

This distribution of antigen was similar to that observed when ferritin-conjugated pig anti-Toxoplasma antiserum (Matsubayashi and Akao, 1966), or mouse anti-Toxoplasma immunoglobulins (Carosi et al. 1980), were used to label ultrathin sections of mouse peritoneal exudate which contained both endozoites and parasites developing within macrophages.

Antigenic differences have been shown to occur between cystozoites and endozoites (Lunde and Jacobs, 1983,1985; Omata et al. 1989). In these studies, antisera raised against endozoites were shown to react with both endozoites and cystozoites. However, antisera raised against cystozoites reacted only with cystozoites, leaving endozoites unstained. The primary layer for the immunogold method used in the present study was a polyclonal anti-Toxoplasma IgG fraction, (Section 2.14.2) This IgG was derived from the immunization of rabbits with killed

endozoites of the virulent RH strain of _Toxoplasma_,
followed by a further inoculation with live _Toxoplasma_ of
the same strain.  Thus the antibody used in the present
study was considered more appropriate for the investigation
of the distribution of _Toxoplasma_ antigen occurring in
association with tissue cysts, than an antiserum raised by
inoculation of suspensions containing _Toxoplasma_ tissue
cysts of cystozoites, since it is likely to reflect more
closely the mode of antibody production in a naturally
infected host.


Light microscope immunocytochemical investigations using
the peroxidase anti-peroxidase technique (Conley and
Jenkins, 1981; Kittas _et al_. 1984; Hay _et al_. 1986) on
brain taken from mice infected with _Toxoplasma_, have
consistently revealed an irregular pattern of tightly
packed, round to ovoid stained structures, within the
tissue cysts; these were previously presumed to be
cystozoites.  The ultrastructural findings from the present
study, indicated that intracystic staining may have
resulted primarily from the matrix antigen.  In the present
study relatively little antigen was detected in association
with cystozoites, and when present, the distribution
favoured the cytoplasm rather than the plasma membranes of
the parasite. This observation was similar to that of
previous electron immunocytochemical studies (Matsubayashi
and Akao, 1966; Carosi _et al_. 1980), where _Toxoplasma_

antigen was not detected on the surface of intramacrophagic parasites.

The light microscope PAP method also demonstrated dense staining delimiting the cystozoites from what was presumed to be the host neuropil. Findings from the present study clearly indicated that this heavy deposition of antigen was confined to the interior aspect of the parasitophorous vacuole membrane.

Small amounts of antigen were detected in the microtubular-neurofilamentous layer, which, as shown in Section 3.2, comprised the host-cell component of the intact tissue cyst "wall" in the brain of the murine model under investigation. Weak deposition was also seen in the neuropil, immediately adjacent to this outermost part of the tissue cyst.

The ultrastructural demonstration of Toxoplasma antigen exterior to the parasitophorous vacuole membrane was reminiscent of the findings using indirect immunofluorescence labelling of intact tissue cysts in mouse brain (Huldt, 1971), where Toxoplasma antigen appeared to `leak' from intact tissue cysts into surrounding brain tissue. If such antigen does `leak' from intact tissue cysts, it does not provoke any obvious cellular inflammatory response in the immediate vicinity of

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**Figure 68:**   Electron micrograph of a disintergrating

_Toxoplasma_ tissue cyst in the brain of a

congenitally infected mouse. Note the

disruption of the cyst "wall" with release

of cystozoite and associated lymphocytes

migrating into the cyst interior.

Mag X4800.
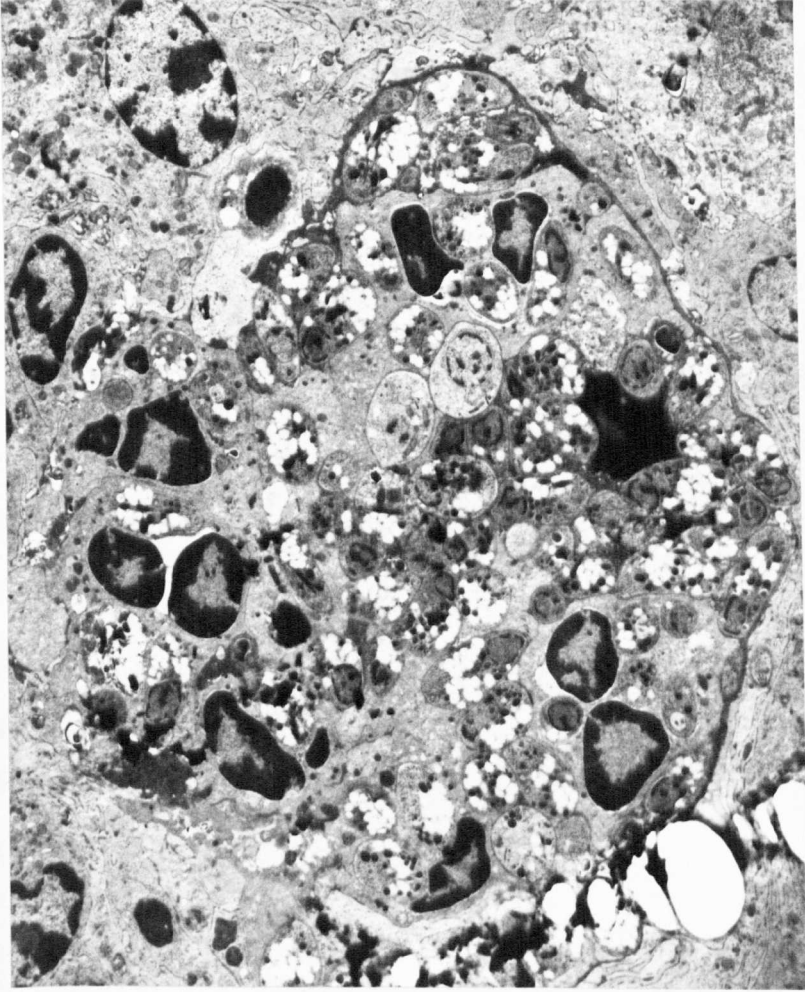
Figure 68:

histological studies of mouse (Hay et al. 1986) and human brain (Conley et al. 1981).

As a consequence of tissue cyst disruption, matrix antigen and cystozoites would be presented to the host immune system. The matrix antigen may represent the large amounts of PAP positive debris observed in foci of mononuclear inflammatory cells in mouse (Conley and Jenkins, 1981, Kittas et al. 1984; Hay et al. 1986), and humans brain (Conley et al. 1981). The observation that some cystozoites within the intact tissue cysts did not react with the anti-Toxoplasma IgG could provide a possible explanation for the persistence of parasitaemia during Toxoplasma infection, a phenomenon which as been observed in both laboratory animals and humans (Remington and Krahenbuhl, 1982).

## 4.4    VASCULAR STUDIES

There has recently been considerable interest in the UK regarding the screening for anti-Toxoplasma antibodies in the pregnant woman (Joss et al 1990). The contention is that early detection of infection would permit chemotherapeutic intervention, thus preventing, or diminishing, the untoward effects of transplacental transmission of the parasite (Desmonts, 1990).

Congenital _Toxoplasma_ infection can manifest in two ways in the newborn infant: there can be a range of readily detectable clinical signs and symptoms with well-defined pathological changes, or the infection can present no obvious clues to its presence (Remington and Desmonts, 1990). The latter group of infected individuals are of considerable interest, since it has been demonstrated that they are likely to develop ocular and/or neurological sequelae in later life (Wilson _et al_. 1980; Koppe _et al_. 1986).

It is clearly difficult to investigate the underlying pathogenesis of congenital toxoplasmosis in such individuals. Thus an animal model of the congenital disease has been developed (Hay _et al_. 1981), in order to characterize the neuro-ophthalmic (Graham _et al_. 1984), and muscular pathology (Hay _et al_. 1988), in the clinically inapparent form of _Toxoplasma_ infection.

It is likely that congenital toxoplasmosis progresses or recrudesces as a result of events involving, either directly, or indirectly the _Toxoplasma_ tissue cyst (Frenkel, 1988). Inflammatory cell infiltrates which produce the pathology in the brain and elsewhere may be attracted to antigen derived from these structures (Remington and Krahenbuhl, 1982). In order to gain access to the tissue, the inflammatory cells must first pass through the small vessels in the vicinity of the tissue cyst.

Thus, in the present study electron microscopy and light
microscope immunocytochemistry were used in order to
examine the pathological changes associated with small
vessels in the proximity of intact <u>Toxoplasma</u> tissue cysts
in the brains of mice with congenital <u>Toxoplasma</u> infection,
which was clinically inapparent.

The observations described in Section 3.5.1, showed that in
small vessels in the vicinity of intact <u>Toxoplasma</u> tissue
cysts, there was sequestration of various inflammatory cell
types, especially lymphocytes and macrophages, into the
perivascular region. The extent of small vessel pathology
ranged from a minimal inflammatory cell infiltrate to an
oedematous change with extensive thickening of endothelial
basment membrane.

In these inflammatory areas, T cells were observed to
predominate, although B cells were invariably present.

Based on the observations presented in Section 3.5.2. it is
tempting to speculate that this recruitment of inflammatory
cells into the perivascular region does not occur in a
random manner and that it actually occurs as follows: B
cells first, followed by T cells and macrophages.

The central nervous system does not have a lymphatic
drainage system. Thus, lymphocytes and other inflammatory
cells entering the brain must do so directly from the blood
stream. Similarly, any processed or unprocessed antigens
must leave the brain by the same vascular route. It has
been shown in Section 3.4.1, that there is leakage of

Toxoplasma antigen from intact tissue cysts in the brains of congenitally infected mice; and, Toxoplasma is known to produce soluble antigens (Saavedr et al.1990). Such antigen might be expected to traverse the neuropil into the region of small blood vessels. Such antigen was not detected using the methods described in the present study; this might be the result , however, of insensitivity of the procedure. This antigen could effectively induce mobilization of previously committed lymphocytes (Remington and Krahenbuhl, 1982). However it is known that such cells become localized in perivascular areas as a result of interaction with extracellular matrix protein such as laminins, collagen and fibronectins. The cells would remain in the vicinity as a result of adhesion molecules (Anon, Lancet 1990); this process is known to occur in the human central nervous system (Sobel 1990).

Events such as immunosuppression or malignancy can result in disruption to extracellular matrix-adhesion components of inflammatory cells (Pignatelli, 1990). This might permit migration of the previously committed lymphocytes along a chemotactic concentration gradient towards the intact Toxoplasma tissue cyst, contained within its functioning host neurone (see Section 3.2).

Such cells in contact with the host component of the cyst "wall" would release lymphokines, thus bringing about the disintergration of the microtubular element of the host cell(Figure 68). The parasitophorous vacuole membrane which

is antigenic to the host (Kimata, 1987), along with matrix antigens (see Section 3.4.1), would then be presented directly to the host immune response.

This hypothetical series of events, offers a novel explanation of how intracystic Toxoplasma may be released from intact tissue cysts into the brain tissue to initiate the neuropathological lesion associated with progressive congenital toxoplasmosis (Beverley 1971).

## 4.5. IMPLICATIONS FOR CHEMOTHERAPY AND IMMUNOTHERAPY FOR CONGENITAL TOXOPLAMOSIS

The findings from the present study are of considerable importance with regard to the treatment and prophylaxis of toxoplasmosis. The consequences of Toxoplasma infection in the immunocompromised patient and in the congenitally infected infant can be very severe.

There are several drug regimes available to the clinician, but none can be said to be 100% effective in the eradication of Toxoplasma in the host. The most favoured regime is the combination of pyrimethamine and sulphadiazine, which is effective against endozoites (Eyles and Colman 1955; Ruskin and Remington 1976; Remington and Desmont 1976). In the immunocompromised patient however, these drugs are relatively ineffective; Levi et al. (1985) reported a mortality rate of greater than 70% of

immunocompromised patients treated with these drugs for
toxoplasmic encephalitis. Moreover, the toxicity of these
drugs on bone marrow production tends to preclude their use
during the early stages of pregnancy (Price and Bond, 1973;
Kaufmann and Geisler, 1968).

In Europe, the macrolide antimicrobial drugs of the
erythromycin series are being developed for use in
congenital toxoplasmosis. Spiromycin has been recommended
by Desmont and Couveur (1974); this drug has been known for
some time to be active against acute murine toxoplasmosis,
(Garin and Eyles 1958). More recently, other macrolides
have been explored in a murine model of acute toxoplasmosis
(Remington and Desmonts 1990).These macrolide
antimicrobials are less toxic than pyrimethamine and
sulphadiazine, and have the advantage that they act upon
intracellular organisms.

Thus the findings presented in this thesis especially those
related to the continued intracellular location of intact
Toxoplasma, and that in the murine model of congenital
toxoplasmosis the parasitised cell is always the neurone,
may be considered important as regards the  chemotherapy of
toxoplasmosis. To be totally effective in the treatment of
the infection, drugs must be able to penetrate intact
tissue cysts and inactivate the large pool of potentially
infective cystozoites contained therein.

The low toxicity of the macrolide group and other novel
approaches to chemotherapy (Huskinson-Mark et al. 1991)

will encourage pharmacologists to develop drugs which are capable of entering parasitized cells to elicit a radical cure for both acute and chronic episodes of _Toxoplasma_ infection.

The observations presented in Ssection 3, suggesting that cystozoites may not be especially antigenic to anti-_Toxoplasma_ antibodies raised in the host, is of considerable importance with regard to the development of a suitable vaccine for immunotherapy. The use of immunotherapy for human and animal toxoplasmosis treatment has been the subject of considerable debate for many years,and many workers are currently employed in attempting to develop a satisfactory vaccination procedure against the parasite. However, most studies involve the use of endozoites or cystozoites,.Clearly, immunotherapy aimed against epitopes of these forms of _Toxoplasma_ might be inappropriate, if the disease is prolonged, since the actual disease may result from a cellular immune response to soluble antigens exuding from the intact tissue cyst, and not from _Toxoplasma_ cystozoites.


## 4.6.     SUGGESTIONS FOR FURTHER WORK


A further investigation could be undertaken in order to determine the immunological nature of the soluble antigen `leeched' from intact tissue cysts of _Toxoplasma_ using a macrophage-_Toxoplasma_ _in-vitro_ system. The macrophage plays

an important role in the establishment of a <u>Toxoplasma</u>

infection in the host and also in the subsequent

pathogenetic process. Thus the macrophage provides an

excellent model for such an investigation; also a

considerable amount of data is available concerning the

association between these phagocytic cells and <u>Toxoplasma</u>.

Supernatant from cultivated macrophage monolayers infected

with <u>Toxoplasma</u> could be collected and subjected to

immunological analysis. After varying incubation times,

samples of culture supernatant could be taken and either:

a) subjected to immunoelectrophoresis; b) used to raise

rabbit anti-<u>Toxoplasma</u> antibody for use in electron

immunohistochemical studies; c) used for lymphocyte

transformation studies; d) used for macrophage

inhibition/migration studies.

Such investigations would provide further information

regarding the role of the intact tissue cyst in the

pathogenesis of subclinical congenital toxoplasmosis.

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