TISSUE CULTURE OF COCCIDIA

by

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Thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy of the
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January 1980

Abstract

Sporozoites of Eimeria tenella were grown in cell cultures of host and non-host tissues. Poor development in non-host cultures may have been due to the obligatory maintenance at 37°C or to absence of the necessary depth of tissue. Second generation schizonts and gametocytes occurred regularly in chick kidney and embryonic chick kidney and liver cells. Other cells supported only first generation schizonts. Complete development with oocysts was seen on one occasion only in chick kidney cells. Morphology and sizes of these stages were compared with results obtained by other workers. No development occurred when merozoites of E. necatrix were inoculated onto host or non-host cells.

Fewer cells were invaded by sporozoites of E. tenella when invasion took place in the presence of new born calf, donor horse serum or protease inhibitors. Inhibition was partially removed by acid or heat treatment of the serum. Chicken serum, normal and inactivated had little effect.

Cells in the S phase of the host cell's cycle were preferentially invaded by sporozoites. Host cells containing developing parasites were more rounded, and showed loss of cell – cell contact and changes in the cell – substrate adhesion patterns. In coculture with parasitised cells the non-parasitised cells mimicked these alterations and showed enhanced ^{3}H-thymidine uptake compared with non-parasitised cells in monoculture. Parasitised cultures showed enhanced agglutinability with Concanavalin A which was eliminated by pre-fixation.

Cell surface glycoproteins were labelled with ^{3}H-1-fucose: the loss of radioactivity from cell preparations and the gain in radio-activity by the supernatants from parasitised and non-parasitised cultures was compared. An increase in radioactivity in the super-natant over parasitised cells was attributed largely to the sloughing off of cell fragments from the cells and this "contamination" obscured the possible release of glycopeptides from the cell coat. Differences in loss of radioactivity from parasitised cells and non-parasitised cells was first observed 2 days post inoculation with sporozoites.

Acknowledgements

Contents

List of Figures

List of Figures (cont.)

List of Figures (cont.)

List of Figures (cont.)

List of Tables

SECTION 1: INTRODUCTION

1:1   Normal Life-Cycle of Eimeria

The sporozoan genus <u>Eimeria</u> contains a large number of species

parasitic in vertebrate animals of all classes throughout the world,

although none infects man. A few species have been recorded, some

rather doubtfully, from annelids, arthropods and protochordates

(Pellérdy 1974). With other genera, including <u>Isospora</u>, <u>Sarcocystis</u>

<u>Toxopla na</u> and <u>Besnoitia</u> they form the Suborder Eimeriina, often referred

to colloquially as the coccidia. <u>Eimeria</u> species are obligate intra-

cellular parasites, spending the multiplicative stage of their life cycle in the cells of

one host. The transmissive stage is a thick walled oocyst, which in

the majority of species enters and leaves the host via the alimentary

canal. This stage is capable of surviving long periods outside the host.

Most species parasitise the epithelial cells of the intestinal villi but

occasionally can be found in deeper tissue. <u>E. steidae</u> develops in the

liver of rabbits (Pellérdy 1974) and <u>E. neitzi</u> causes uterine coccidiosis

in the impala (McCully, Basson, de Vos and de Vos, 1970).

The infection begins when the sporozoite excysts from the oocyst,

the excystation process varying slightly with the host. In ruminants

the oocysts probably undergo the first phase of excystation in the rumen

(Jackson 1962). Here the micropyle is split, lifted or thinned by the

action of carbon dioxide. In avian hosts the oocysts spend little time

in the crop and the effect of carbon dioxide is considered to be minimal

(Long and Speer 1977). In gallinaceous birds mechanical damage to the

oocyst by the gizzard is probably essential for sporocyst release.

Sporocysts of <u>Eimeria</u> species have a thickening at the anterior end

called the Steida body. When this comes into contact with trypsin and

bile salts in the intestine it is digested, the sporozoites then
leaving via the resulting pore. Recently it has been shown that
chymotrypsin, a contaminant of commercial trypsin, is the essential
enzyme for excystation (Wang and Stotish 1975). The freed sporozoites
then invade the intestinal mucosa. The rate of excystation varies
considerably between species and could determine the site of development
(Long and Speer 1977). The chicken coccidia, E. praecox and E. acervulina
both excyst within a few minutes and develop in the upper small intestine.
E. tenella develops in the large intestine and sporozoites can take up
to 2 hours to excyst.

Multiplication within the cell proceeds by an asexual process
called schizogony. The resulting merozoites are released and must
re-invade new cells to initiate a second generation. The number of
generations varies between species but eventually the merozoites enter
cells and instead of developing into schizonts they develop into sexual
individuals or gametocytes. The female or macrogamete remains uninucleate.
The male or microgametocyte undergoes repeated nuclear division to
produce a large number of flagellated, motile microgametes. These are
released, swim towards the macrogamete, penetrate the host cell and
achieve fertilisation. The resulting zygote encysts whilst still in the
host cell. Within this wall the sporozoites are formed although the
process of sporulation usually begins outside the host. The nucleus
divides meiotically and four sporoblasts are produced. These then encyst
within the oocyst as sporocysts, their contents dividing to produce the
sporozoites. When this differentiated oocyst is ingested by another
individual the life cycle begins again.

Most species in the Eimeriina have similar life-cycles, differing in minor ways such as the number of sporocysts and sporozoites per oocyst, the number of asexual generations and the development site in the host. Eimeria oocysts have 4 sporocysts each with 2 sporozoites, Isospora oocysts have 2 sporocysts each with 4 sporozoites. Some members of this Suborder have more than one host e.g. Sarcocystis.

The rearing of domestic animals under intensive conditions provides an abnormally high number of hosts over a small area - ideal conditions for a parasite to spread. Such heavy infections by Eimeria spp. produces the disease coccidiosis, recognised as of veterinary importance. It is known to occur in many domestic animals such as chickens, turkeys, rabbits, cattle and sheep (Levine 1973). Heavy infection with the more pathogenic species causes death, mainly due to the heavy blood and fluid loss caused by excessive damage to the intestinal wall.

1.2 Tissue Culture of Eimeria

Consideration of the economic consequences of coccidiosis, stimulated study of the parasite, with a view to producing prophylactic drugs. Observations were hampered by the fact that the parasites were intracellular for much of their life-cycle. With the development of cell culture a system became available which allowed the parasite to remain visible throughout its life cycle and had the added advantage that, as the immediate environment could be controlled, the behaviour of the parasite could be studied under a variety of conditions.

1.2.1 Development of Chicken Coccidia in Tissue Culture

i Sporozoite Inoculum

The development in tissue culture of Eimeria species from chickens,

from a sporozoite inoculum, is listed in Table 1.1. The sporozoites

entered a wide variety of cells, but in some of them they underwent

little further development. Completion of the life cycle of E. tenella

was first achieved in vitro by Long (1965) when he inoculated sporozoites

into the chorioallantoic membrane (CAM) of embryonated chicken eggs. In

the same year Patton (1965) reported the development of mature first

generation schizonts of E. tenella in cultures of bovine kidney cells

and Japanese quail embryo fibroblasts. Doran (1970a)first reported the

completion of the life-cycle of E. tenella in cultures of kidney cells

derived from adult and embryonic chickens. The oocysts produced were

infective to birds not previously in contact with any coccidial infections.

Subsequently the production of E. tenella oocysts has been reported in

chick kidney cells by several workers: Strout and Ouellette (1970);

Klimes, Rootes and Tanielian (1972); and Itagaki, Hirayama, Tsubokura,

Otsuki and Taira (1974).

Limited success has been achieved with other coccidia from chickens.

There appeared to be a relationship between the degree of development in

tissue culture and the site of development in the host, the most complete

development occurring with species having all or part of their life-cycle

in the large intestine (Ryley and Wilson 1972a). Thus E. brunetti, which

develops in the rectum, was grown to mature second generation schizonts

in a variety of cells (Table 1.1.); E. necatrix, which has its third

schizogony and gametogony in the caeca, grew to mature second generation

schizonts in primary cultures of chick kidney (Table 1.1.); E. maxima,

Table 1.1 Development of Eimeria spp. from chickens in cell culture from a sporozoite inoculum

Species	Cells[a]	Type of Culture[b]	Extent of development[c]						Reference
			Intra-cellular Sporozoite	Troph-ozoite	imma-ture schizonts	mature schizonts	gametes	oocysts	
E.acervulina	Chick kidney(E)	Primary	✓	1					Strout et al 1965 Itagaki et al 1974
	Chick Kidney	Primary	✓ ✓			4			Doran 1971a Itagaki et al 1974 Naciri-Bontemps 1976
	Chick Fibroblast(E)	Primary	✓	1					Strout et al 1965 Itagaki et al 1974
	Mouse Fibroblasts (L cells)	CL		1					Strout et al 1965
	Human Amnion	CL		1					Strout et al 1965
	Human Cervical Carcinoma (HeLa)	CL		1					Strout et al 1965
	Human Kidney (E)	CL	✓						Doran & Vetterling 1967b
	Bovine Kidney (E)	Primary and CL	✓						Doran & Vetterling 1967b
	Ovine Kidney	Primary and CL	✓						Doran & Vetterling 1967b
	Porcine Kidney	Primary and CL	✓						Doran & Vetterling 1967b
E.brunetti	Chick Kidney	Primary			2	2			Ryley & Wilson 1972a Itagaki et al 1974
	Chick Fibroblasts (E)	Primary				2 2			Shibalova 1970 Itagaki et al 1974
	Chick Kidney (E)	Primary				1			Itagaki et al 1974
	Quail Fibro-blasts (E)	Primary				2			Shibalova 1970

Species	Cells	Type								Reference
E.necatrix	Chick Kidney	Primary				2				Doran 1971a
	Bovine Kidney (E)	CL				1				Doran & Vetterling 1967b
		Primary	✓							Doran & Vetterling 1967b
	Human Kidney (E)	CL	✓							Doran & Vetterling 1967b
	Ovine Kidney	Primary and CL	✓							Doran & Vetterling 1967b
	Porcine Kidney	Primary	✓							Doran & Vetterling 1967b
		CL			1					Doran & Vetterling 1967b
E.tenella	Chick Kidney (E)	Primary				2				Shibalova 1968
								✓		Strout & Ouellette 1969
								✓		Shibalova 1970
							!		✓	Doran 1970a
									✓	Strout & Ouellette 1970
	Chick Kidney	Primary							✓	Doran 1970a,1971a,b
									✓	Klimes et al 1972
									✓	Itagaki et al 1974
	Chick Fibroblasts (E)	Primary				1				Bedrnik 1969a
						2				Shibalova 1968,1969 Shibalova et al 1969
					1					Patton 1965
	Quail Fibroblasts (E)	CL				1				Patton 1965
	Mouse Fibroblasts (L cells)	CL			1					Patton 1965

Species	Tissue	Type							Reference
	Bovine Kidney	Primary				1			Ryley 1968
		CL (MDBK)				1			Patton 1965
	Bovine Kidney (E)	CL				1			Doran 1969,1970a
	Bovine Trachea (E)	CL				1			Matsuoka et al 1969
	Human Fibroblasts	CL				2			Shibalova 1968,1969,1970
	HeLa	CL				2			Shibalova 1969,1970
	Monkey Kidney	CL				2			Shibalova 1969
	Partridge Kidney	Primary						✓	Doran 1971b
	Pheasant Kidney	Primary							✓ Doran 1971b
	Turkey Kidney	Primary				2			Doran 1971b
E.maxima	Chick Kidney	Primary	✓						Doran 1969,1971a
E.mivati			✓						
E.praecox			✓						

a : E = Embryonic   b : CL = Cell Line   c = number denotes the generation

E.mivati and E. praecox, all parasites of the small intestine, entered

chick kidney cells but developed no further (Table 1.1.). E. acervulina,

a parasite of the small intestine, has generally failed to develop beyond

the trophozoite stage in cell culture. However, Naciri-Bontemps (1976)

reported the development of mature fourth generation schizonts in chick

kidney cells. Long (1973) mentioned that this distinction between the

abilities of species of Eimeria from chickens to grow in cell culture

also applied to the ease with which they infected and grew in the CAM of

embryonated eggs.

ii Merozoite Inoculum

Merozoites, obtained from chickens, have also been used to achieve

development of Eimeria species in cell cultures. The results of those

experiments are shown in Table 1.2. Oocysts of E. tenella have been

produced in this manner (Bedrnik 1967b, 1969a,b, 1970; Shibalova 1970).

Also, by using second generation merozoites, the life-cycles of E. tenella

in cell culture were extended to produce a third (Bedrnik 1967a) fourth

(Bedrnik 1967b) and maybe a fifth (Bedrnik 1969b) generation of schizonts.

Although E. acervulina showed little or no development when sporozoites

were used as an inoculum (Table 1.1.), with fourth generation merozoites

obtained from axenic chickens, Naciri-Bontemps (1976) grew oocysts in

primary chick kidney cells.

1.2.2 Development of Eimeria Species from Non-Avian Hosts in Tissue Culture

The results of culturing non-avian coccidia in vitro, from a

sporozoite inoculum are listed in Table 1.3. All species tested,

achieved a considerable degree of development in both host and non-host

cells.

Table 1.2 Development of <u>Eimeria</u> species from chickens in cell culture from a merozoite inoculum

Species	Cells[a]	Type of Culture[b]	Source of Merozoites	ND[d]	Troph-ozoite	Schizont[e] imm.	Schizont mat.	Gam[f]	Oocyst	Reference
<u>E.acervulina</u>	Chick Kidney	Primary	Host(4)[c]					✓	✓	Naciri-Bontemps (1976)
<u>E.brunetti</u>	Chick Fibroblasts (E)	Primary	Host(2)					✓		Shibalova 1970
	Quail Fibroblast (E)	Primary	Host(2)					✓		Shibalova 1970
<u>E.tenella</u>	Chick Fibroblast (E)	Primary	Host(2)				3[c]			Bedrnik 1967a
									✓	Bedrnik 1967b 1969a 1969b
						4/5			✓	Bedrnik 1969b
				✓						Itagaki <u>et al</u> 1974
	Chick Caecum & Liver	Primary	Host(2)						✓	Bedrnik 1969b 1970
	Chick Kidney (E) and Adult	Primary	Host(2)	✓						Itagaki <u>et al</u> 1974
	Quail Fibroblast (E)	Primary	Host ?						✓	Shibalova 1970
	Cervical Carcinoma (HeLa)	CL	Host(2)				3			Bedrnik 1967a 1969b 1970

<u>Key:</u>
a: E= Embryonic
b: CL = Cell Line
c: Number denotes generation
d: ND = Intracellular, no development
e: imm = immature
   mat = mature
f: gam = gametocyte

Table 1.3 Development of non-avian _Eimeria_ in tissue culture (sporozoite inoculum)

Species	Cell Type							References
	Bovine Kidney CL(MDBK)	Bovine Kidney (E) CL[a]	Bovine Intestine (E)[b] CL	Bovine Trachea (E) (EBTr)	Human Intestine CL(Int 407)	Ovine Kidney (E)	Ground Squirrel Embryo	
CATTLE _E.alabamensis_	[c]Troph[2]	[d]Mat.schzt[1][f]	Mat.schzt[1]	Mat.schzt[1]	Mat.schzt[1]	–	–	Sampson et al 1971
E.auburnensis	Mat.schzt[1]	–	–	Mat.schzt[1]	Troph[1]	–	–	Clark & Hammond 1969
E.bovis	Mat.schzt[1]	Mat.schzt[1]						Hammond & Fayer 1967
				Imm.schzt[2][e]	Mat.schzt[1]			Hammond & Fayer 1968
UNITA GROUND SQUIRREL _E.bilamellata_	Mat.schzt[1]	Mat.schzt[1]	–	Mat.schzt[1]	Imm.schzt[1]	–	–	Speer, Hammond & Anderson 1970
		Mat.schzt[1]	Mat.schzt[1]	–	–	–	–	Speer, Hammond & Kelley 1970
E.callospermophili	Mat.schzt[1]	Troph[2]	–	Mat.schzt[1]	Mat.schzt[1]	–	Imm.schzt[2]	Speer, Hammond & Anderson 1970
	–	Mat.schzt[1]	Mat.schzt[1]	–	–	–	–	Speer, Hammond & Kelley 1970
E.larimerensis		Mat.schzt[1]	Mat.schzt[1]	–	–	–	–	Speer, Hammond & Kelley 1970
	Imm.schzt[2]	–	–	–	–	Imm.schzt[2]	Imm.schzt[2]	Speer & Hammond 1970
SHEEP _E.ninakohlyakimovae_		Mat.schzt[1]	Mat.schzt[1]	–	–	–	–	Speer, Hammond & Kelley 1970
	Mat.schzt[1]	–	–	–	–	Mat.schzt[1]	–	Kelley & Hammond 1970
RAT _E.neischulzi_	–	Mat.schzt[1]	Mat.schzt[1]	–	–	–	–	Speer, Hammond & Kelley 1970
RABBIT _E.magna_	Mat.schzt[2]	N/D[g]	–	–	–	–	–	Speer & Hammond 1971

a : CL = Cell Line
b : E = Embryonic
c : Troph. = Trophozoite
d : Mat.schzt = Mature schizont
e : Imm.schzt = Immature schizont
f : Number denotes generation
g : N/D = no development

1.2.3 Other in vitro Systems for the Growth of Eimeria

The entire life-cycle of E. tenella was completed when sporozoites were inoculated onto the chorio-allantoic membrane (CAM) of embryonated eggs (Long 1965, Shibalova 1969a, 1970). No development in this system occurred when the CAM tissue was inoculated with E. acervulina or E. maxima sporozoites although late schizonts of E. necatrix and oocysts of E. mivati and E. brunetti were obtained (Long 1965).

CAM tissue, previously inoculated with E. tenella sporozoites, was trypsinised and plated out, after a few days of growth of the parasite had occurred. Using this method Long (1969) was able to produce oocysts. He stated that although the numbers of oocysts produced was low compared with the numbers of gametocytes present, they sporulated at room temperature and produced infection when fed to chickens.

1.2.4 The Life-Cycle and Development of Eimeria spp. in vitro

i Asexual Generations

Once intracellular, the sporozoite becomes enveloped in a membrane-bound parasitophorous vacuole, and enlarges to form the trophozoite. This transformation is usually achieved by a gradual increase in width following an initial widening of the anterior tip. Subsequent upon this in E. tenella, are two asexual generations or schizogonies, as in vivo. Strout and Ouellette (1970) found that the merozoites of E. tenella became spontaneously activated when they had separated from the parent schizont but still remained within the parasitophorous vacuole. They also mentioned that their release did not occur simultaneously; some merozoites escaped by penetrating an extremely elastic membrane, others were released after 2-3 hours of activity, when the membrane was eventually torn or broken down. Most of these merozoites invaded cells within the

vicinity of the parent schizont immediately after their release, or degenerated (Strout and Ouellette 1970). Second generation schizonts matured in a similar fashion to the first generation although they were often smaller by virtue of the multiple invasion of cells. (Strout and Ouellette 1970).

Shibalova (1974) reported the asynchronous development of merozoites within schizonts of E. tenella growing in tissue culture. Some nuclei in a schizont were involved in the budding of merozoites whilst others were not. Lee and Millard (1971) reported a similar feature with E. praecox in the host.

ii Sexual Generation

The second generation merozoites of E. tenella in cell culture usually develop into the gametocytes. This occurs 6 days after inoculation of chick kidney cells (Doran 1970a; Strout and Ouellette 1969, 1970; and Itagaki et al 1974) or at 8 days in embryonic chick cells (Doran 1970a). The macro- and micro-gametocytes are usually observed clustered together with several in the same cell. Itagaki et al (1974) described them as often being within a common parasito-phorous vacuole, and suggested that as the merozoites were harboured very close together this common vacuole was probably produced by fusion of neighbouring vacuoles as the gametocytes grew.

Oocysts were produced in chick kidney cells on the sixth (Doran 1970a, 1971b,c,) and seventh (Itagaki et al 1974) days post-inoculation of cultures with E. tenella sporozoites.

iii Third Generation Schizogony

At the time that the gametocytes of E. tenella appear small mature

schizonts which contain only a few merozoites can be observed

(Doran 1970a; Strout and Ouellette 1970). Because of this timing

and their location next to immature and mature second generation

schizonts they are believed to represent the third generation. Such

a generation has been described in vivo for E. tenella (Tyzzer 1929).

1.2.5 The Morphology of the Asexual Generations of E. tenella in vitro

Long (1969) described four basic types of schizonts of E. tenella

grown in cells from CAM tissue, cultured a few days following

parasitisation. Schizonts resembling the in vivo stages occurred

(Types 1 and 4 representing first and second generation schizonts

in vivo, respectively). However, there was a preponderance of Types

2 and 3. In these latter schizonts, merozoites were arranged in

rosettes around a residual mass. They either occurred as one solid

schizont (Type 2) or in groups, producing the smallest schizonts grown

(Type 3). Occasionally a parasitophorous vacuole contained numerous,

separate masses of material (blastophores), from the periphery of which

the merozoites were later seen to form. Types 2 and 3 were smaller and

contained fewer merozoites than the conventional schizonts (Types 1 and 4).

Strout and Ouellette (1970) described the first and second

generation schizonts of E. tenella as exhibiting one of two morphologies.

The conventional schizont, which resembled the in vivo situation had

merozoites arranged in a rosette shape. The second type they termed

blastophore schizonts: in this type the protoplasm was subdivided into

distinct spheroidal bodies, with merozoites forming in each. Although

both types were typical of first and second generation schizonts, third

generation schizonts were always rosette-shaped.

Itagaki et al (1974) described three basic types of schizonts of
E. tenella found in chick kidney cells. Type I they equated with the
in vivo conventional schizont and Type II with the blastophore schizont
of Strout and Ouellette (1970). Type III they compared with the
sporozoite-shaped schizonts described for other species, in vivo and
in vitro.

1.2.6 The Morphology of the Asexual Generations of other Eimeria species in vitro.

Blastophore schizonts have often been recorded from cultures of
other species. They have been described for E. bovis (Fayer and
Hammond 1967; Hammond and Fayer 1968) E. auburnensis (Kelley and
Hammond 1970) and E. crandallis (de Vos, Hammond and Speer 1972).
Although blastophore schizonts are not known to occur in natural
infections of any avian coccidia they are a normal stage of development
of E. bovis (Hammond, Ernst and Miner 1966) and E. auburnensis (Chobotar
Hammond and Miner 1969).

The sporozoite-shaped schizonts described for E. tenella by Itagaki
et al (1974) were also seen in culture forms of other species, occurring
when nuclear division preceded the transformation to a trophozoite or
schizont. The number of nuclei before this transformation was 2-5 in
E. auburnensis (Clark and Hammond 1969), 2-8 in E. magna (Speer and
Hammond 1971), 2-12 in E. callospermophili and 2-8 E. bilamellata (Speer,
Hammond and Anderson 1970). Although Itagaki et al (1974) said that the
fate of E. tenella sporozoite-shaped schizonts was unknown, details have
been provided for other species. Clark and Hammond (1969) described this
transformation for E. auburnensis, which took place when a marked bulge
appeared at the end/middle of the body of the schizont, the resulting
spheroidal schizont developing within seconds.

The behaviour of these schizonts is very interesting. Those of

E. callospermophili are motile and able to leave and enter host cells,

indicating that they have retained the structures of the sporozoites

specialised for movement and penetration (Speer, Hammond and Anderson

1970). This was confirmed by ultrastructural observations on

E. callospermophili sporozoite-schizonts (Roberts, Hammond, Anderson

and Speer 1970). All the organelles representative of the motile stages

were present i.e. conoid, pellicle, subpellicular tubules, rhoptries and

micronemes. Similar movement has been observed in this schizont type for

E. alabamensis (Sampson, Hammond and Ernst 1971) E. larimerensis (Speer

and Hammond 1970; Speer, Davis and Hammond 1971) and E. magna (Speer and

Hammond 1971).

1.2.7 Comparison of in vitro with in vivo development

i Timing of Life-Cycles

The timing of the life-cycle of E. tenella in vitro shows more

variation than in vivo. In a review of the cultivation of coccidia in

cell cultures, Doran (1973) mentioned that E. tenella developed faster

at the beginning of its life-cycle in vitro than in vivo. However,

development gradually became slower, so that with the appearance of the

gametocytes and oocysts the timing in the host and in adult chick kidney

cells was similar. In embryonic chick kidney cells and those cells

derived from non-host tissue oocysts appeared later than they would in

the host. Long (1965) described delayed development in CAM tissue and

said that it was caused by an extended schizogony. He later attributed

this extended schizogony to a lower incubation temperature (38.6°C) than

E. tenella would have encountered in the host (41°C), (Long 1969).

Hammond and Fayer (1968) described E. bovis development as being
faster in certain types of cells than in calves. Trophozoites appeared
at 3 days instead of 5 days post-inoculation, and mature schizonts
appeared at 8 days, instead of 12 days post-inoculation, when grown in
bovine tracheal cells.

ii Relative Sizes of Developmental Stages

Table 1.4 illustrates the sizes of various stages recorded for the
in vitro development of E. tenella. Generally the first generation
schizonts were smaller in vitro, with fewer (but longer) merozoites than
in vivo. In the second generation both the schizonts and the merozoites
were smaller in vitro than in vivo. The smaller number of merozoites in
this second generation was influenced in part by the multiple invasion of
the host (Strout and Ouellette 1970).

Differences have been recorded with other Eimeria species. Clark
and Hammond (1969) discussed the sizes of schizonts and merozoites of
E. auburnensis grown in vitro, noting that although there was a greater
variation in size in the in vitro schizonts, the overall size of both
schizonts and merozoites was smaller than those found in calves.

Size variations in culture are possibly related to the type of cell
used to cultivate the parasite (Doran 1973). For example, schizonts of
E. meleagrimitis varied in size according to the cell type in which they
were grown (Doran and Vetterling 1968). In embryonic turkey intestine
cells the schizonts were very small and contained only 12-28 merozoites.
A similar situation occurred with bovine kidney cells up to 72 hours
post-inoculation. However, after 72 hours in bovine kidney cells large
schizonts began to appear, which reached greater dimensions and contained
more merozoites than recorded in the host.

Table 1:4   Measurements of developmental stages of <u>E. tenella</u>

	References: cell culture						References: chickens		
	Patton 1965	Strout and Ouellette 1969	Matsuoka <u>et al</u> 1969	Strout and Ouellette 1970	Doran 1970a	Itagaki <u>et al</u> 1974	Tyzzer 1929	Pellerdy 1974	Madden and Vetterling 1978
Mature first generation schizont	–	–	28-37 (diameter)	8x11-35x50	37x30	31.4x18.6	24.0x17.3	–	–
First generation merozoite	7-8 (length)	–	3-5 (length)	7 (length) (200)b	7x1-1.5 (200-250)	3.6x1.5 (20-200)	2.5x1.3 (900)	–	–
Mature second generation schizont	–	–	–	12x15-60x65	42x19	27.3x20.8	43.5x31.0	21-30 (diameter)	21.5-12.5 (diameter)
Second generation merozoite	–	–	–	12-14 (length) (4-200)	12x1.4 (200)	9.3x1.6 (11-150)	16.0	16x2	8.5x1.7 (37-250)
Mature third generation schizont	–	–	–	–	15.0 (diameter)	–	9.0x7.6	9.0x7.6	6.3-11x4.2-7
Third generation merozoites	–	–	–	10.0 (length)	12.0x1.4 (5-17)	–	6.8x1.0	6.8x1.0	3.3x0.57 (5-17)
Macrogametes	–	22.8x12.6	–	22.8x16.5	–	12.5x9.2	–	–	–
Microgameto-cytes	–	20.0x19.6	–	20.0x17.0	–	19.2x11.5	12.4x8.7	–	–

a = all measurements in microns

b = figures in brackets is the number of merozoites/schizont.

1.2.8 The Presence of an Activating Factor in Cell Culture.

i Sporozoites

Although penetration by sporozoites of Eimeria spp. appears to be non-specific in vitro (Tables 1.1 and 1.3) the situation regarding their further development is quite different. Various cell types differ in their ability to support development of a parasite and conversely parasites differ in their abilities to develop within a cell. Doran and Vetterling (1967b) suggested that the absence of a cellular factor could explain the failure of E. necatrix to develop in certain cells. A similar explanation was proposed for E. bovis (Hammond and Fayer 1968). Doran (1971a) suggested that various species of chicken coccidia probably differed in an inherent mechanism which had to be activated by the host cell before development could begin. Itagaki et al (1974) expanded this idea with the suggestion that the three more pathogenic species (E. brunetti, E. necatrix and E. tenella), which grew more easily in cell culture, possessed similar characteristics which enabled them to begin development and that these differed somewhat from those of the less pathogenic species. They suggested that the relevant traits could include a greater ability to adapt to the in vitro environment.

ii Merozoites

Merozoites, as well as sporozoites, are evidently dependent on the presence of an activating substance for further development (Hammond, Fayer and Miner 1969). Bedrnik (1970) obtained greater development of E. tenella merozoites in culture following "misintroduction" of cells from the caeca of infected chicks. He concluded that these cells contained a cellular growth factor which was not present in similar cells from uninfected birds. Doran (1973) suggested that the evidence for such

a factor was small as Bedrnik had failed to take into account i) the confluency of the cell layer, and ii) whether the number of invading sporozoites was proportional to dosage. These are two criteria which must be considered before comparisons of infectivity can be made (Doran 1971c).

Long and Speer (1977) mentioned that the merozoites of E. tenella were more difficult to maintain in a viable condition than sporozoites, regardless of whether they were obtained in vivo or in vitro. Despite gentle handling, and the use of low concentrations of bile salts to stimulate activity, the number invading cells was low. They concluded that bile (or bile salts) were probably only one of several substances required for further in vitro development of coccidian parasites.

However, at least with E. tenella, any involvement in vivo of bile, or bile salts, in further development of merozoites appears unlikely as these substances are not present in the caecum of chickens where merozoites are released and develop.

1.2.9 Conditions of Culture

With the successful completion of the life-cycle of E. tenella from sporozoite to oocyst in culture (Doran 1970a) attempts were made to identify ideal conditions for development.

i Temperature

The optimum temperature for in vitro production of avian coccidial oocysts was found to be 41°C (Doran 1970a), although schizogony was reported to occur between 35-43°C (Strout, Ouellette and Gangi 1969a).

ii Inoculum

Strout, Ouellette and Gangi (1969b) suggested that inoculum size

could affect development. They found that an inoculum of 100,000 sporozoites/ml gave the best results in cell cultures at 96 hours. Doran (1971c) mentioned that a similar size gave the best production of oocysts at 7 days, whereas inocula above 250,000/ml caused a rapid decline in oocyst production.

Patton (1965) reported that débris, such as oocyst and sporocyst walls, in the inoculum could be toxic to cells. Consequently, most workers removed this débris by passing the sporozoite suspension over glass beads prior to inoculation, a method which Wagenbach (1969) had shown to be effective. Alternatively, the uncleaned inoculum could be removed after 24 hours and replaced by fresh medium. With E. meleagrimitis, E. necatrix (Doran and Vetterling 1967a and b) and E. adenoides (Doran 1970b) ineffective removal of débris after inoculation of the cultures resulted in the appearance of dead and dying kidney cells and a noticeable failure of the parasite to develop.

Doran (1971c) found that a cleaned inoculum resulted in fewer intracellular sporozoites at 4 hours, but that the subsequent yield of oocysts at 7 days was greater, than when an uncleaned inoculum had been used. As the condition of the cells at 7 days was the same, regardless of the type of inoculum used, this greater yield of oocysts was attributed to the condition of the sporozoites surviving the passage over the glass beads, rather than to the toxicity of the débris. Fayer and Hammond (1967) mentioned that although the débris sometimes hindered the observation of E. bovis development in culture, it did not appear to have any appreciable toxic effect upon the cells.

iii Type of Cell

Species of Eimeria usually developed best in cells derived from the natural host (Doran 1971b, Itagaki et al 1974). Host cells themselves exhibit variation in their ability to support oocyst production, E. tenella oocysts developing better in adult rather than in embryonic kidney cells (Doran 1970a). Most species prefer to develop in islets of epithelial cells, and development in such areas is thought to be necessary for gametogony (Bedrnik 1967b; Strout and Ouellette 1969, 1970; Doran 1970a, 1971c). These islets develop from aggregates of cells in the initial cell suspension; the numbers of aggregates being related to the trypsinisation procedure (Doran 1970a). Doran (1971c) suggested that the depth of tissue provided by these islets was just as critical as their epithelial nature for the development of the parasite. The failure to develop beyond a certain stage in vitro may merely have been due to the deterioration with time in the condition of the cell layer (Doran 1973).

iv Medium

Initially the medium used in cell culture was believed to have no influence on the parasite's development (Patton 1965), but subsequent studies have shown that medium and medium supplements could affect the invasion and development of Eimeria spp in vivo. Doran (1971c and 1973) demonstrated such an effect during the later part of the life-cycle. Differences between groups grown in various conditions became noticeable only at 4-5 days. However, this could have been due to better conditions for cell growth rather than to a direct effect on parasite development. Thus, favourable conditions could be encouraging the development of the epithelial cells at the expense of the fibroblast population.

Doran and Augustine (1976) compared the oocyst production of

E. tenella in primary chick kidney cells grown under a wide variety

of conditions. They achieved greatest yields by changing the medium

after inoculation. Cells were established in Hank's Balanced Salt

Solution (HBSS) with 10% foetal calf serum and 10% lactalbumin

hydrolysate (LAH,2.5% solution in HBSS). After inoculation of the

sporozoites the medium was changed to one of Basal Medium (Eagle's),

Minimum Essential Medium (Eagle's) or Medium 199, with 5% foetal calf

serum and 5% LAH. Several other workers have mentioned the need for a

separate medium for growth and maintenance of cultures (Doran 1970a,

1971c and Itagaki et al 1974). Itagaki et al (1974) demonstrated that

the addition of a yeast extract benefited the development of E. tenella,

but not E. brunetti, in cell culture.

1.2.10 Uses of the in vitro System of Parasite Development

One potential use for the cell culture is that it provides a method

for the screening of drugs in an economical way (Strout and Ouellette

1971, 1973; McDougald and Galloway 1973). Alternatively, this system has

been used to i) test the degree and site of activity of known anti-

coccidials and ii) to locate that part of the life-cycle where such

drugs are most effective (Fayer and McLoughlin 1970; Ryley and Wilson

1971, 1972b,c, 1975, 1976a,b). By a suggestion that the host cell was

influencing the production of false negatives or positives, Ryley and

Wilson (1976a) called into question the validity and reliability of

in vitro anti-coccidial drug screening methods. However, they felt that

cell culture had a use in the evaluation of the mode of action of

established drugs. Support for the unreliable nature of the in vitro

screen comes from work by Latter and Wilson (1979). They demonstrated

that the differences in reports concerning the effectiveness of aprinocid

as an anti-coccidial drug in vitro were due to the varying metabolic

abilities of the cells to convert it to an active compound. Chick
kidney could not, but chick liver could. In vivo this drug has
anti-coccidial properties. They concluded that this discrepancy, which
was due entirely to the type of host cell used, was a good reason again
to limit the in vitro drug screening method to analysing the effects of
known anti-coccidials.

There have been few reports concerning nutrient requirement for
the development of Eimeria spp., but the in vitro method could provide
a more controllable means of studying this. Sofield and Strout (1974)
studied amino acid requirements and found that media deficient in any
one of glutamine, isoleucine, methionine, tryptophan or tyrosine prevented
the development of the second asexual generation. Ryley and Wilson
(1972b) used the cell culture system in a series of growth antagonism
studies. They suggested it provided a means of learning more about the
basic nutrients required by Eimeria spp., although they admitted that the
problem of permeability barriers and host metabolism could not be
eliminated. Doran and Augustine (1978), investigating the micronutrient
requirements for the development of E. tenella in tissue culture, found
that schizogony depended on the presence of several vitamins. However,
they stressed that the situation pertaining in vivo could be different,
and the actual micronutrient requirement of E. tenella only resolved when
the parasite was grown axenically in chemically defined medium.

1.3 Invasion of Cells

The invasion of cells is crucial to the life-cycle of all coccidia.
The development of the in vitro system has enabled the invasive process
to be studied in a more controlled manner with a view to understanding
the mechanisms involved. Most of the work has been concerned with
sporozoites.

1.3.1 Movement of Sporozoites

When inoculated onto cell cultures sporozoites are extremely active and begin invading cells within seconds. The movements they perform are characteristic of the various species and are listed in Table 1.5.

TABLE 1.5   Movement of excysted sporozoites when inoculated

on to cell cultures

Movement	Species	Reference
Flexing	E.alabamensis	Sampson et al 1971
Gliding	E.auburnensis	Clark and Hammond 1969
Gliding and Flexing	E. larimerensis E..boris	Speer and Hammond 1970 Fayer and Hammond 1967
Gliding, Flexing and Pivoting	E.callospermophili E.bilamellata E.larimerensis E.tenella E.meleagrimitis	Speer and Hammond 1969 Speer, Hammond & Anderson 1970 Speer, Hammond & Anderson 1970 Speer, Davis & Hammond 1971 Fayer 1972 Fayer 1972
Helical	E.acervulina	Doran et al 1962
Gliding and Helical	E.larimerensis	Roberts et al 1971

Prior to entry, sporozoites of E. auburnensis (Clark and Hammond 1969), E. tenella and E. meleagrimitis (Fayer 1972) perform gliding movements, whereas those of E. larimerensis about to enter cells undergo gliding and helical movements (Roberts et al 1971). Changes also occur at the anterior tip. Roberts et al (1971) described these changes in detail in E. larimerensis. The sporozoites inserted a slender protuberance into the host cell to a depth of 1-2µ. This was repeatedly inserted and retracted at different locations within the immediate vicinity. Usually the protuberance moved laterally for 1-2 seconds and appeared to swell by

increasing in width and decreasing in length. Within 3 seconds of

these changes the remainder of the sporozoite had penetrated the cell.

Speer et al (1971) showed with ciné studies that this protuberance was

withdrawn before the parasite entered the cell. Fayer (1972), in

describing gliding sporozoites of E. meleagrimitis and E. tenella,
                                                             that
said the anterior tip changed from a blunt point to a marked acumination or

spinous projection. Penetration occurred when this spinous projection

had formed.

Only rarely is host cell cytoplasm seen to escape as sporozoites

enter cells. (Roberts et al 1971, Speer et al 1971). It is more

frequently observed as they leave (Speer et al 1971), which suggests

that this is a much more disruptive process than entry.

1.3.2 The Apical Complex of Motile Stages

i Ultrastructure

Sporozoites and merozoites of Eimeria are characterized by the

presence of an apical complex at their anterior end. Many workers have

suggested that the organelles comprising this complex are involved in

invasion, (Roberts and Hammond 1970; Ryley 1969; Scholtyseck, Mehlhorn

and Friedhoff 1970). Basically the complex consists of a conoid, which

is a truncated hollow cone of 6-8 spirally arranged fibrils. Anterior

to the cone are one or more preconoidal rings. Attached to the posterior

ring are the subpellicular microtubules which radiate outwards and extend

backwards for almost the entire length of the body. Scholtyseck (1973)

mentioned that the number of these differed in the various developmental

stages of coccidia; in sporozoites and merozoites of many Eimeria species

there were 24. The club-shaped organelles or rhoptries also originate

from the conoid and extend back into the body of the sporozoite and

merozoite. Lying between the microtubules and the rhoptries are the

micronemes. Detailed ultrastructural studies have been made of this

structure (Scholtyseck et al 1970; Scholtyseck and Mehlhorn 1970;

McLaren and Paget 1968; Ryley 1969; Roberts and Hammond 1970).

ii Role in Invasion

McLaren and Paget (1968) postulated a reversible extrusible/

retractable role for the conoid in the merozoites of E. tenella.

They suggested that it played a part in the entry of cells by

merozoites, and that the rhoptries, for which a secretory role was

postulated, were also involved. This idea was supported by a micro-

graph which showed empty rhoptries in a merozoite which was actively

penetrating a cell. McLaren and Paget (1968) and Ryley (1969) noticed

a pore at the anterior end of the conoid of merozoites and sporozoites,

respectively, of E. tenella. This indicated a possible route by which

the rhoptry contents were secreted when the conoid was applied to the

host cell membrane.

Scholtyseck et al (1970), when describing the change in shape of

the anterior tip of sporozoites during penetration, suggested that mechanical

entry was involved. They postulated that the subpellicular microtubules

could push the conoid out by contracting, and that the polar rings might

act as a vice preventing the conoid emerging too far. Lysogenous enzymes,

produced by the rhoptries, would be squeezed out by the same contractions

and possibly aid invasion.

In a later review of Sporozoan ultrastructure, Scholtyseck and

Mehlhorn (1970) suggested that the rhoptries were only excretory

ductures for substances (possibly proteolytic enzymes) produced in the

micronemes. Furthermore the fibrillar wall of the rhoptries was

responsible for discharging the contents of this functional system. The

enzymes aided mechanical perforation by the conoid and affected the host cell cytoplasm in such a way as to produce an environment more suitable for the parasite.

1.3.3 Mechanism of Entry of Eimeria

Initially mechanical puncturing of the membrane was generally held to be the mode of entry into cells, by both sporozoites and merozoites. Later work disproved this and showed that the membrane was invaginated, rather than ruptured, at the site of entry. Following this the nature of the rhoptry-microneme system was studied in order to find out how this invagination was initiated and/or maintained.

i Mechanical Entry

Fayer and Hammond (1967) described E. bovis sporozoites as entering cells rapidly, which suggested to them that a mechanical puncturing of the membrane was involved. They did not exclude the possible involvement of lytic enzymes. Ryley (1973) described the conoid in sporozoites and merozoites of Eimeria species as acting like a captive bolt or pistol to achieve a puncturing of the membrane. Other species initially thought to enter by a mechanical means were described: E. auburnensis (Clark and Hammond 1969), E. bovis (Fayer and Hammond 1967), E. ninakohlyakimovae (Kelley and Hammond 1970), E. larimerensis (Roberts et al 1971, Roberts and Hammond 1973), E. callospermophili (Roberts, Hammond and Speer 1970) and E. alabamensis (Sampson et al 1971).

Ultrastructural studies appeared to confirm this idea. Roberts et al (1971) showed by electron microscopy that sporozoites of E. larimerensis fixed in the process of entering a cell were constricted at the site of entry and the host cell membrane was seen to have partly invaginated. As it still appeared interrupted at the site of entry they concluded that

mechanical penetration occurred, but only after an initial invagination. This invagination provided a portion of the membrane lining the vacuole.

ii Lysing the host cell membrane

Doran and Vetterling (1967b) observed sporozoites of E. acervulina undulating slowly back and forth and pulling at the cytoplasm before they entered. This suggested to them that the sporozoites had lysed the membrane to effect entry.

Müller (1975) showed that E. contorta entered cult~~ured~~ ivared cells rapidly, with a local disruption of the outer membrane occurring during penetration. He felt that the sporozoites actively invaded the cells. The parasitophorous vacuole was formed afterwards by the merging of several small vacuoles apparent around the parasite after invasion.

iii Invagination of the Host Cell Membrane

The evidence available now is overwhelmingly in favour of a non-disruptive invagination of the host cell membrane during penetration. Jensen and Hammond (1974), although initially favouring a phagocytic mode of entry for E. magna into cult~~ured~~ ivared cells, later modified their ideas. In 1975, Jensen and Hammond finally showed, by electron microscopy, that the sporozoites entered by an active process, and the membrane of the host was not broken during invagination. The invagination was sealed off by short pseudopodia after the parasite had worked itself deeply into the cell. This left the sporozoite inside a completely membrane-bound vacuole and explained why host cell cytoplasm was rarely observed to escape during penetration. The host cell membrane lining the vacuole broke down soon after invasion and was replaced by another of host cell origin. Jensen and Edgar (1976), in ultrastructural studies of E. magna invasion, subsequently provided evidence that the rhoptry secretions had effected this breakdown of the membrane. During

invasion, the invaginating host cell membrane became frayed and diffuse where it lay adjacent to the apical tip of the sporozoite. After penetration was complete this disintegration progressed posteriorly until the parasite lay completely free in the cytoplasm of the host cell. Concurrent with these alterations was the appearance of empty or partially empty membrane saccules in the apical region of the sporozoite. These saccules were of the same shape, size and location as the rhoptries. Often discrete packets of osmiophilic material, analogous to the rhoptry contents, were seen aligned in channels which passed through the conoid. They therefore proposed that the empty saccules represented the remnants of the rhoptries which, being contractile, had released their contents during penetration.

1.3.4 Mechanism of entry by other Sporozoa

A great deal of the recent advances in the understanding of invagination have come from studies on the invasion of erythrocytes by Plasmodium spp. merozoites. Plasmodium yoelii and P. gallinaceum enter cells by causing an invagination of the membrane (Ladda, Aikawa and Sprinz, 1969). The vacuole forms by the meeting of cytoplasmic constrictions and not from short pseudopodia as in E. magna (Jensen and Hammond 1975) and the host cell membrane remains intact. Ladda et al (1969) stressed the possible role of the rhoptries in secreting a substance to aid invasion, because early in the process of entry they became less dense and rapidly disappeared. Similar results were obtained for P. knowlesi merozoites (Bannister, Butcher, Dennis and Mitchell 1975) and although the participation of rhoptries, micronemes and microspheres were implicated they believed these organelles remained separate throughout the invasive process.

Therefore, it is apparent that there is a basic mechanism of invasion in the Sporozoa, and Bannister (1977) drew attention to two areas of similarity. Firstly, other genera invade by causing invagination e.g. Babesia (Rudzinska 1976), and Toxoplasma (Jones, Yeh and Hirsch 1972; Klainer, Krahenbuhl and Remington 1973). Secondly, in all the above genera the apical complex is in contact with the zone of invagination. Bannister (1977) therefore proposed that the apical complex was producing a membrane-expanding material, as in Plasmodium. Aikawa and Sterling (1974) denied the involvement of rhoptry/microneme secretions in invasion. However, Bannister (1977) felt that the evidence available weighed in favour of rhoptry involvement and cited, amongst other results, the dense material resembling rhoptry contents which Jensen and Hammond (1975) had demonstrated inside the invagination during its formation. Bannister (1977) concluded by suggesting that the remaining contents of the rhoptry/microneme system were discharged after invagination to enlarge the vacuole. Recent results from invasion studies on Isospora canis (Jensen and Edgar 1978) have served to confirm a common mechanism of entry.

1.3.5 Chemical Nature and mode of action of the Rhoptry-microneme secretions

i Enzymatic

Garnham, Bird and Baker (1960) suggested, in their description of the rhoptries of Haemamoeba ($\equiv$ Plasmodium) gallinacea sporozoites, that they may be producing a proteolytic enzyme to facilitate entry into the host cell. Subsequent analysis in this, and other Sporozoan genera has been limited. Most work has been done by a group of workers from Sweden on the related organism Toxoplasma gondii. Lycke, Lund and Strannegård(1965) found that two proteases, hyaluronidase and lysozyme, enhanced the penetration of T. gondii merozoites into cells. Lycke and

Norrby (1966) extracted a factor from lysed parasites which enhanced penetration. This penetration-enhancing factor (PEF) was selectively directed against the host cell wall (Norrby and Lycke 1967) and was said to resemble lysosomal enzymes. The kinectics of PEF synthesis was related to host cell penetration (Norrby 1970). Furthermore, using immunofluorescence tests Norrby (1971) showed that the production of PEF was associated with the rhoptries. However, the idea that it may resemble lysosomal enzymes has recently been questioned (Lycke, Carlberg and Norrby 1975), despite their original observations (Lycke et al 1965). This conclusion was drawn from the fact that the amount of lysosomal enzymes tested which was necessary to achieve effects comparable to those of PEF was considerably greater in all cases. However, they still believed that invasion involved an enzymatic process. Hyaluronidase has been shown to be absent in T. gondii (Timofeev 1971), and the involvement of this enzyme in Eimeria invasion has also been questioned (Fayer, Romanowski and Vetterling, 1970). These authors attributed the inhibition of entry of E. adenoides into cells by hyaluronidase to the increased viscosity produced at high concentrations.

Quinine in the inoculation medium prevented the invasion of sporozoites of Eimeria (Fayer 1971), T. gondii, Besnoitia jellisoni and Sarcocystis sp. (Fayer, Melton and Sheffield 1972). No visible cytopathological effects were observed; neither were the sporozoites rendered immotile. The above authors suggested that quinine could have been acting by inhibiting enzymatic action, or by changing the surface of the cultured cells. They concluded by postulating the involvement of esterolytic enzymes.

ii <u>Polycationic Polypeptides</u>

The concept that the secretions of the rhoptries are not actually proteolytic in nature has gained increasing support over the last few years. Kilejian (1976) isolated a histidine-rich polypeptide from <u>P. lophurae</u> merozoites which was associated with the rhoptries and micronemes. At points of contact between the membrane and large aggregates of the protein, distinct invaginations occurred. De Souza and Souto-Padrón (1978) likewise demonstrated basic proteins in <u>T. gondii</u> using a stain which binds mainly to proteins rich in lysine, arginine and histidine. The properties of this protein did not accord with those of PEF isolated by Lycke <u>et al</u> (1975), and so de Souza and Souto-Padrón (1978) concluded that there was more than one invasive component present in <u>T. gondii</u>.

Although such a protein has not been isolated from any <u>Eimeria</u> species, Jensen and Edgar (1976) suggested that the evidence was in favour of its presence. They supported this by drawing attention to work by Shotton, Elgsaeter and Bronton (1975) on erythrocyte membranes. Shotton <u>et al</u> (1975) demonstrated that polycationic polypeptides produced aggregations of protein constituents in erythrocyte membranes which lead to the appearance of numerous vesicles and blebbing from an incompressible lipid layer. As blebbing and vesicle production along the invaginating membrane accompanied invasion by <u>E. magna</u> they suggested this similar sequence of events indicated the presence of such polypeptides (Jensen and Edgar 1976). Alternatively events similar to those described for <u>P. knowlesi</u> invading merozoites (see following section) could be involved.

iii Differential Expansion of Plasma Membrane Bilayer and Incorporation

of Parasite Products

Ladda et al (1969) suggested that the rhoptry secretions produced

changes at the surface of the cell. Recently Bannister et al (1975)

suggested that the inward expansion of the erythrocyte membrane could

be attributed to the differential incorporation in the lipid bilayer

of the rhoptry and microneme product.

Disruption of Plasmodium rhoptries and treatment with metallic

stains releases a multilamellate material (Bannister, Butcher and

Mitchell 1976). Similar effects occur with invading E. magna

sporozoites fixed with glutaraldehyde containing ruthenium red (Jensen

and Hammond 1975). Bannister (1977) mentioned that although such

appearances may reflect the binding of metals to the rhoptry contents

rather than to their normal state of organization, the fact that they

produced lamellae when released into an aqueous medium suggested that

they might possess the ability to form lamellae under other circumstances.

Furthermore, McLaren, Bannister, Trigg and Butcher (1979) demonstrated

membranous lamellae and vesicles in the vicinity of invading P. knowlesi

merozoites. These were structurally similar to those observed in the

sectioned material of Bannister, Butcher and Mitchell (1977). The

decrease in numbers of intramembranous particles (IMP) described for

membranes of developing parasitophorous vacuoles is consistent with the

idea that there is a "flooding" effect as these membranous lamellae are

incorporated (McLaren et al 1979).

1.3.6 The Role of Phagocytosis in Sporozoan Invasion

Although phagocytosis has been suggested as a means of entry for

Eimeria spp. (Doran and Vetterling 1967b; Strout, Solis, Smith and

Dunlop 1965) it has never actually been recorded. Jensen and Edgar (1976)

observed invasion in the presence of antiphagocytic agents. Micro-
tubule disruptors did not affect invasion or motility of the sporozoites.
Cytochalasin B, a microfilament disorganizer, lowered the invasion rate
by interfering with sporozoite motility. They therefore concluded that
entry into cells by the parasite depended more on sporozoite motility
than on any action of the cell.

Transmission electron microscopy (Jones et al 1972) and scanning
electron microscopy (Klainer et al 1973) showed micropseudopods
surrounding T. gondii as it entered cells. As these micropseudopods were
apparent in phagocytic and non-phagocytic cells, Jones et al (1972),
suggested the secretions of the parasites had induced phagocytosis.
Lycke et al (1975), however, proposed that phagocytosis played no part
in the invasion of T. gondii because i) T. gondii was equally infective
for phagocytic and non-phagocytic cells, ii) toxoplasmas were only
engulfed and digested after they had been killed, and iii) neuraminidase
(which enhanced phagocytosis in monocytes) had no effect on the infection
rate of T. gondii.

1.3.7 Merozoite and Microgamete Motility and Invasion

Long and Speer (1977) suggested that merozoite invasion by Eimeria
was very similar to that by sporozoites. They also postulated the involve-
ment of enzymatic action in the release of merozoites from the schizonts.
Mature merozoites of E. magna which were still retained in the schizont
had large vesicles at the anterior tip, immediately anterior to the conoid.
This was the point where the merozoite surface made contact with the
vacuolar membrane, suggesting that these vesicles contained enzymes
which facilitated their release. Long and Speer (1977) mentioned that
several substances were probably required for the activation and release

of these merozoites.

Virtually no studies have been made on the release and invasion of microgametes of Eimeria. E. tenella microgametes, once freed from the host cell in which they matured, move by means of two flagella to seek the macrogamete (Madden and Vetterling 1977). The perforatorium, an organelle at the anterior end, may be concerned in the penetration of the macrogamete (Long and Speer 1977).

1.4 Host Cell Changes

Preliminary work was limited to describing the cytopathological effects of parasite invasion and development. In later work, some advances were made on the ultrastructural and biochemical nature of these alterations. Most advances have been made with Plasmodium spp.

1.4.1 Cytopathological Alterations

With the formation of the parasitophorous vacuole there is hypertrophy of the host cell cytoplasm both in vivo and in vitro. Intestinal cells infected with E. necatrix showed a profound increase in size (Fernando and Pasternak 1977). In vitro, cells infected with E. meleagrimitis and E. necatrix schizonts became 2-3 times larger (Doran and Vetterling 1967b), and those infected with E. tenella schizonts 8-10 times larger (Patton 1965), than normal.

Vacuolisation of the cytoplasm often accompanies these changes (Bedrnik 1969b; and Scholtyseck 1969). In E. bovis (Fayer and Hammond 1967) and E. ninakohlyakimovae (Kelley and Hammond 1970) the cytoplasm forms a thin layer around the schizont except in the vicinity of the host cell nucleus. Cytoplasmic vacuolisation is indicative of cell degeneration. In some cases (E. auburnensis) it gradually spreads throughout the culture to affect non-infected cells during the later

stages of the parasites' development, with finally the whole monolayer

degenerating (Clark and Hammond 1969). These authors mentioned that

this could be due to the effects of a toxic product of the parasite.

Alternatively, deterioration in the condition of the cultures could be

caused by the rupture of cells as the merozoites are released (Matsuoka,

Callender and Shumard 1969; Sampson et al 1971).

Doran and Vetterling (1968) noted that cells infected with

sporozoites or mature schizonts of E.meleagrimitis became partially or

completely detached from the surface of cultures. Similar results are

seen with E. tenella but not E. brunetti (Itagaki et al 1974).

1.4.2 Damage to Host Cells in vivo

Gross morphological changes occur in the intestines of birds

infected with Eimeria (Pellérdy 1974). Scanning electron microscopy

has revealed that excessive infection of chickens with Eimeria spp.

produces a sloughing off of the epithelial layer, resulting in the

extrusion of the underlying lamina propria (Witlock and Ruff 1977).

E. brunetti caused complete destruction of the villus and the villar

epithelium, whereas E. acervulina caused dramatic 'clubbing' of the

villi, and E. mivati infection resulted in only a slight shortening.

1.4.3 Ultrastructural Changes

Work concerned with the ultrastructural changes in host cells has

been mainly descriptive.

i Cytoplasmic alterations

Finger-like projections of the host cell cytoplasm extend into

the vacuole (Sampson and Hammond 1972; Shibalova 1974). These folds

very often become disconnected from the membrane (Hammond, Scholtyseck

and Miner 1967) disintegrating to form particulate matter which can be

ingested by the parasite (Scholtyseck 1973; Kelley and Hammond 1972).

Numerous mitochondria become located in the host cell cytoplasm adjacent to the membrane lining the parasitophorous vacuole (Sampson and Hammond 1972). This same area is also almost devoid of ribosomes. These authors felt that the association of the mitochondria with the membrane indicated energy drainage. Toxoplasma causes a similar "overcoating", with mitochondria and endoplasmic reticulum (ER) accumulating around the parasitophorous vacuole of macrophages within minutes of invasion (Jones and Hirsch 1972). In this case it was suggested to result from a protective mechanism on the part of the host cell; alternatively it may be serving a nutritional role for the parasite.

E. tenella causes an accumulation of rough ER, mitochondria and Golgi vesicles around the parasitophorous vacuole (Shibalova, 1974). This occurs immediately after the formation of the vacuole. The mitochondria could represent a change in cell energy transport, whereas the Golgi vesicles and rough ER were probably involved in the formation of the new membrane. Rough ER is the predominant site of membrane protein and phospholipid synthesis, with the carbohydrates being added in the Golgi vesicles (Quinn 1976).

ii Parasitophorous vacuolar membrane alterations

Ladda et al (1969) reported that during the entry of avian malarial merozoites into erythrocytes the invaginating plasma membrane eventually lined the parasitophorous vacuole. Trager (1972) showed that treatment of the erythrocytes by several methods released the parasite still bound by these two membranes, i.e. its own plasma membrane and the membrane of the parasitophorous vacuole. P. lophurae merozoites released in this way from duck erythrocytes showed no reaction with anti-serum to duck erythrocytes. They did agglutinate with serum from hyperimmune ducks, which had no effect on the duck erythrocytes. This indicated a

change in the plasma membrane once it formed the lining of the parasitophorous vacuole.

With E. magna a complete replacement of the parasitophorous vacuole membrane occurs (Jensen and Edgar, 1976) soon after the vacuole is sealed. No studies have been performed to investigate the resemblances this may have to the former membrane or to the contemporary plasma membrane of the host cell.

Recently, McLaren et al (1979) have extended the work on Plasmodium in this respect. By freeze-fracture studies they showed that the IMP pattern of the new parasitophorous vacuole membrane was unique. Initially it was devoid of any IMPS but their gradual reappearance during the development of the parasite, they thought, represented the intercalation of parasite proteins. Furthermore, the reversed polarity of the IMP at the schizont stage tied in with cytochemical studies. Langreth (1977) had shown that certain enzymes were located on the opposite sides of the membrane from that expected if it was merely an invaginated erythrocyte plasmalemma.

With other sporozoan genera similar changes would appear to be taking place. Jones and Hirsch (1972) described changes to the membrane of the parasitophorous vacuole surrounding T. gondii which were due to secretions of the parasite. These resulted in the inability of the lysosomes to fuse with the vacuolar membrane. However, this was a localized change as only dead parasites in cells containing both dead and live toxoplasmas were subjected to lysosomal attack.

Other protozoal parasites which develop in macrophages avoid lysosomal attack in other ways. Chang and Dwyer (1978) showed that Leishmania donovani amastigotes did not prevent fusion of lysosomes with the parasitophorous vacuole. The amastigotes, however, managed to

maintain their ultrastructural integrity for up to two weeks despite
the presence of lysosomal enzymes in the phagosome. Chang and Dwyer
(1978) felt that this lent evidence to their previous theory (Chang and
Dwyer 1976) that survival was due to the refractory properties of the
amastigotes' surfaces to lysosomal degradation, rather than to the release
of any enzyme inhibitors. They mentioned further that the parasite's
surface had glycoproteins which could bind the lysosomal hydrolases
and thereby inactivate them.

Noqueira and Cohn (1976) described how Trypanosoma cruzi trypo-
mastigotes avoided lysosomal attack. The phagosome membrane completely
disappeared releasing the parasite into the cytoplasm, where development
took place without a surrounding vacuole. The authors suggested that this
membrane had been lysed by a factor released by the parasite or by some
more direct membrane - membrane interaction.

iii Host Cell Plasma Membrane Alterations

Circumstantial evidence exists that plasma membranes of host cells
are altered when infected with Eimeria spp. in vivo. Fernando and
Pasternak (1977) described a method for the isolation of E. necatrix
infected cells. By using hypotonic buffer they selectively lysed non-
infected cells and suggested that modifications to the membranes of
infected cells had prevented a similar response. They mentioned
unpublished results which suggested that E. tenella produced a similar
effect. However, as there was no difference in the response of
parasitised and non-parasitised CAM tissue to homogenisation with the
buffer they concluded that any membrane modifications were tissue-specific.

Hermann (1969) demonstrated that extracts of erythrocytes infected
with P. lophurae or extracts from free parasites increased the osmotic
fragility of normal, washed duck erythrocytes. McLaren et al (1979)

suggested a connection between this and the fall in IMP levels as recorded by freeze-fracture.

iv Alterations to Host Cell Nuclei

In vivo nuclear hypertrophy of infected cells has led to incorrect identification of the host cell (Gresham and Cruickshank 1959). As a result of the gross distortion of the host cell's nucleus these authors described the second generation schizonts of E. tenella as occurring in macrophages. Bergmann (1970) showed by electron microscopy that these cells were epithelial by origin. Scholtyseck (1953) described these changes in vivo as being noticeable when the sporozoite was small and far from the nucleus.

In vitro, although such changes become more pronounced in cells containing the later stages of the parasites (Fayer and Hammond 1967), they are in evidence much earlier. Most species produce nuclear hypertrophy except some of those infecting the Unita ground squirrel (Speer, Hammond and Anderson 1970). These species cause a decrease in size of the host cell nucleus, with an accompanying development of an irregular outline. These changes are evident within an hour of inoculation with E. callospermophili. In cells infected with E. bilamellata they only become noticeable after the formation of the merozoites, at which time the nucleus is generally 1/3-1/2 the normal size.

Nucleoli enlarge (Fayer and Hammond 1967; Kelley and Hammond 1970; de Vos et al 1972) and the chromatin often becomes rearranged (Fayer and Hammond 1967; Kelley and Hammond 1970).

Nuclear hypertrophy is accompanied by increases in DNA. A sixfold increase occurs with chick cells infected with second generation schizonts of E. tenella (Beyer and Shibalova 1974). Fernando, Pasternak, Barrell and Stockdale (1974) reported that the hypertrophy associated

with E. necatrix (sexual stages) and E. tenella (second generation
schizonts) was accompanied by an increased content of DNA, to a 4C
equivalent. This was not accompanied by cell division. E. maxima,
however, caused no changes to nuclear size and DNA content in vivo.
Fernando et al (1974) proposed that these different responses were a
reflection of the different properties of the host cells. E. tenella
and E. necatrix both infected crypt epithelial cells. As these had
remained in an undifferentiated state they were able to accumulate
DNA following coccidial infection. E. maxima, however, parasitised
the differentiated cells of the villus, which were unable to respond to
infection in this manner. Fernando et al (1974) concluded that
induction of DNA synthesis occurred in those cells destined under normal
conditions to undergo DNA replication. Further evidence for this is
provided with a study of E. zuernii from cattle (Pasternak, Fernando,
Stockdale and Weber 1977).

The enlargement of nucleoli in cells infected with E. necatrix
schizonts is associated with increases in nucleolar RNA (Fernando and
Pasternak 1977).

In vitro, infection of cells by E. tenella is followed by
unscheduled DNA synthesis, as measured by uptake of ^{3}H-thymidine
(Browning, Patton and Lytle, 1976), it is significant at 24 hours after
inoculation of cultures. It is independent of both the number and
development of the parasite. Unlike the reports in vivo (Fernando et al
1974), Browning et al (1976) showed that the stimulation was transferred
to non-parasitised cells which showed an equivalent increase in uptake
of ^{3}H-thymidine.

v Division of Infected Cells and Tissue

Long (1970a) suggested that in vitro the division of infected cells

was inhibited. <u>In vivo</u> the division of progenitor cells would also be inhibited to allow development of the parasite to proceed undisturbed. However, Fernando and McCraw (1977) demonstrated that the sloughing off of the infected tissue <u>in vivo</u> was accompanied by an increase in the rate of replacement of epithelial cells. The first generation of schizonts developing in the crypt epithelial cells stimulated these progenitor cells to divide faster. The shortening cell cycle time was attributable to a shortened G_1 or pre-synthetic phase.

1.5 Aim of Present Study

The system of tissue culture of <u>Eimeria</u> species has benefited from more than a decade of fairly intensive study. However, as the preceding review has indicated, very little advance has been made to the knowledge of such basic features as invasion and host cell changes. It is important that a full understanding be reached of the means by which <u>Eimeria</u> i) enters the cells, and ii) alters the host cell structure and functions to further its own needs. Until such time, any attempts to develop drugs to control this important disease will be hampered by the lack of much information concerning the parasite's biology. It was the aim of the present study to establish a good and reliable method for growing the parasite in culture. Using this system, it was hoped that a further understanding of the mechanism of <u>E. tenella</u> sporozoite penetration into cells would be achieved. Finally, a more detailed study would be made of the effects the parasite had on its host cell during its intracellular development.

SECTION 2: MATERIALS AND METHODS

2.1 Parasites

Oocysts of Eimeria tenella were kindly supplied by Dr. R.

Williams from Burroughs Wellcome, Berkham ste d, and those of

E. necatrix and E. acervulina by Dr. E. Michael from May and Baker

Ltd., Ongar. They were received and stored in the sporulated

condition at 4°C in a 2.5% solution of potassium dichromate ($K_2Cr_2O_7$)

until required.

2.2 Preparation of sporozoites

Oocysts were washed clean of $K_2Cr_2O_7$ using sterile deionised

water and then resuspended in undiluted sodium hypochlorite and placed

for 10-15 minutes on ice. Although this procedure is known to alter

the outer layer of the oocyst wall (Nyberg and Knapp, 1970), damage to

the internal contents of the oocysts is negligible and consequently it

provides a safe means of preparing sterile cultures of sporozoites.

Sterile deionised water was then added to the tube containing the

oocyst suspension in sodium hypochlorite. By adding the water slowly

it was possible to prevent mixing of the two solutions and after

centrifugation at 2100 r.p.m./10 minutes the oocysts accumulated at the

water/hypochlorite interface. The oocysts were removed, washed several

times in sterile deionised water and finally resuspended in phosphate

buffered saline (PBS) pH7.6, once all traces of the sodium hypochlorite

had disappeared. The sporocysts and sporozoites were released from the

oocysts by a method similar to Long (1970b). The sporocysts were

released by rapid shaking with 0.5mm glass beads on a whirlimix for

about 40 seconds. This suspension was washed free from the glass beads,
 resuspended in trypsin: bile (0.25%: 5%, respectively in PBS, pH7.6), incubated @ 41°C for 40 min — 1 hour, when
 about 80% excystation was obtained. It was then
and spun down at 2500 r.p.m. for 5 minutes, washed with PBS (pH7.6) and

then passed through a glass bead column prepared according to Wagenbach

(1969) except that it was modified to fit into a 5ml syringe. The resulting filtrate was again washed in PBS (pH7.6) and either resuspended in medium for inoculation of cell cultures, or stored at 4°C until needed, (Millard and Long (1974) having shown that sporozoites of <u>E. tenella</u> survived in buffer solution at 4°C for 14 days).

2.3 Preparation of merozoites

The procedure for obtaining merozoites of <u>E. necatrix</u> was essentially similar to that described for <u>E. tenella</u>, by Stotish and Wang (1975). Chickens were fed 15,000 oocysts/bird and sacrificed 5 days later. The lower small intestine was removed, cut open longitudinally and the contents discarded. The tissue was washed with phosphate buffered Ringer's solution (pH7.4), containing 100 units of penicillin and 100μg of streptomycin per ml, and cut into $0.5cm^2$ sections. The sections were placed in 10 volumes of an incubation solution containing 120 mM NaCl, 20mM Tris-Cl(pH.7.4), 3mM K_2HPO_4, 1mM $CaCl_2$ and 1mg/ml bovine serum albumin (B.S.A., Sigma Chemicals Ltd). To this solution was added hyaluronidase to a concentration of 1mg/ml and the mixture was incubated at 37°C with moderate agitation for 30 minutes. Merozoite release was monitored microscopically; some free merozoites could be observed after 20 minutes incubation. The suspension was filtered through sterile gauze to remove large pieces of tissue and then spun down at 1000 r.p.m. for 5 minutes to remove the incubation medium. The resulting pellet was washed twice with sterile Ringer's buffer (pH7.4) and filtered through a glass bead column, prepared as above, to remove the smaller pieces of tissue. The merozoites were added straight away to the cell cultures.

2.4 Tissue Culture

All tissue culture products were obtained from Flow Laboratories, Scotland, except where indicated.

2.4.1 Sterilisation procedure

Glassware and instruments were cleaned in a 2% Decon 90 solution (Decon Laboratories, Ltd.). Coverslips were washed in two changes of absolute ethanol and air dried. Coverslips for reflection interference microscopy were soaked overnight in a solution of Pyroneg (Diversey, Ltd.), washed several times in tap water, three times in distilled water, once with methanol and then air dried. Sterilisation was carried out in an autoclave by moist heat at 15lbs/sq.inch for 20 minutes. Pipettes, coverslips, glass wool, glass beads and scalpel blades were sterilised by dry heat at over 160°C for 2 hours. Tissue culture medium, buffers and enzyme solutions were sterilized by filtration through a sterile 0.22μm filter under positive pressure. A laminar flow cabinet was used for all tissue culture work, which was carried out using aseptic technique.

2.4.2 Trypsinisation of tissue for primary cultures .

2.4.2i Primary cultures of chicken kidney cells

Cultures of chick kidney (CK) cells were obtained by the methods described by Doran (1970a, 1971c). 1-3 week old chickens were sacrificed and opened by a ventral incision. The kidneys were removed and washed in PBS and placed in a Petri dish containing fresh PBS. Excess connective tissue was removed and the kidney tissue separated into 2-3mm^2 pieces. The pieces were placed in a universal tube containing PBS without calcium and magnesium (PBS without Ca and Mg) and shaken vigorously to remove excess blood cells. The washed tissue was added to the trypsinising solution in an Erlenmeyer flask. This trypsinising

solution was 0.25% trypsin (1:250) and 0.02% ethylene diamine tetra-

acetic acid, disodium salt (EDTA) in PBS without Ca and Mg. This

tissue suspension was stirred at 37°C and the supernatants from the

first, second and third trypsinisation periods discarded (10,7 and

5 minutes, respectively). The supernatants of the fourth, fifth and

sixth trypsinisation periods (each 3 minutes long) were pooled and

cold 10% new born calf serum (NBCS) in Hank's Balanced Salt Solution

(HBSS) was added. The resuspended cells were spun down at 200 r.p.m.

for 5 minutes, washed twice in cold 10% NBCS (in HBSS) and resuspended

in the growth medium. This growth medium was HBSS supplemented with

10% NBCS, 2% 1-glutamine, 2.5% of a 10% lactalbumin hydrolysate

solution (L.H.) (Gibco-Biocult, Ltd.), and penicillin/streptomycin

solution to 100 units/ml and 100μg/ml, respectively. The medium

contained phenol red indicator and was adjusted to a pH of 7.4 with

the addition of a 1M HEPES solution (N-2-hydroxyethyl piperazine-N'-2-

ethanesulphonic acid) to a final molarity of 20mM. This buffer negated

the need to gas cultures with a carbon dioxide/air mixture and in no

way hindered the development of the parasite or the growth of the cells.

Kidney tissue from 18 day chick embryos was treated in a similar way to

adult tissue, except that the first trypsinisation period of 10 minutes

was omitted.

For most experiments cells were seeded on to 16mm diameter glass

coverslips placed in the wells of sterile plastic repli dishes (Sterilin,

Ltd.) at known concentrations between 2×10^5 and 6×10^5 cells/well, in

2 mls of growth medium. For scanning electron microscopy cells

(2×10^5/well) were seeded on to 13mm diameter coverslips. For reflection

interference microscopy 32mm diameter, No. 2, glass coverslips were

placed in 35mm Petri dishes (Gibco-Biocult, Ltd.) and between 1×10^6 and

1.5 x 10^6 cells, in 4 mls of growth medium, was added to each dish.

Cells for the agglutination assay were seeded at a concentration of

2 x 10^6 in 5 mls of growth medium into 35mm tissue culture treated

Petri dishes (Gibco-Biocult, Ltd.).

Cultures were placed at 41°C and 24 hours later the growth

medium was removed and replaced with fresh growth medium. Changes of

medium were made regularly until the cells were ready for inoculation

with sporozoites/merozoites, usually 4 or 5 days later.

2.4.2ii Primary cultures of other chick embryonic tissues

The procedure was essentially similar to that described for

kidney tissue in Section 2.4.2i, except embryonic liver was trypsinised

for periods of 5,3,3 and 3 minutes, with supernatant from the 5 minute

period being discarded.

Chick embryonic fibroblast (CEF) cultures for reflection inter-

ference microscopy were prepared following the method of King, Heaysman

and Preston (1979). Explants from 7 day-old chick embryo hearts were

cultured in glass Petri dishes in Medium 199 (M199) with 1% foetal calf

serum (FCS). After 2 days the cells were removed by trypsinisation and

then allowed to settle on to 32mm diameter No. 2 coverslips. The

fibroblasts were inoculated 72 hours later with sporozoites.

2.4.2iii Macrophage cultures from embryonic chickens

The spleen from 17-19 day old embryos was removed and cut into cubes

approximately 1-2mm^2. Three or four of these pieces were then

transferred to 16mm diameter coverslips held in individual wells of

repli dishes (Sterilin, Ltd.). 2 mls of growth medium was added,

composed of M199 (Hank's Salts) supplemented with 10% chicken serum,

but otherwise as for the growth medium described for chick kidney cells

(2.4.2i). After 24 hours the tissue was dislodged and the medium changed.

Medium changes were made at 48 hour intervals thereafter. After 10

days the cultures were usually overrun by fibroblasts.

2.4.2iv Macrophage cultures from the mouse peritoneum

Three days prior to use, a mouse was given 2 mls of a 2% starch

solution intra-peritoneally. After three days the mouse was sacrificed

and the peritoneal cavity was washed out with 50 units of cold heparin

in 5 mls of saline. The suspension was spun down at 600 r.p.m. for 5

minutes and resuspended in cold M199. The pellet was washed twice

with cold M199 and resuspended in M199 with 10% NBCS. The cells were

counted and plated out at 3×10^6 cells/16mm coverslip. The medium

was removed after 2 hours and replaced with fresh growth medium.

Cultures were inoculated with merozoites 24 hours later.

2.4.3 Growth and maintenance of mammalian cells.

2.4.3i Growth and maintenance of cell lines

Cell lines were maintained as described in Table 2.1.

2.4.3ii Preparation of neutrophils

Samples of whole, heparinised blood removed from normal donors was

spun down on a Ficoll-Hypaque gradient. The layer containing the red

blood cells and granulocytes was removed. This pellet was resuspended

in 2.5% gelatine in 154mM NaCl and left at 37°C for 30 minutes. After

this incubation period most of the red blood cells sank to the bottom

and the supernatant was composed mainly of granulocytes (virtually all

the granulocytes were neutrophils). This supernatant was spun down at

200g/1 minute to produce a neutrophil pellet contaminated by some red

blood cells. The pellet was resuspended in 0.83% ammonium chloride

(pH7.4) and left to incubate at 37°C for 5 minutes; this procedure lysed

all contaminating red blood cells. The suspension was spun down again

Table 2.1 The growth and maintenance of cell lines

Cell Line	Origin	Growth medium[a]	Split ratio	Cell Type	Temperature	Passage No.
Embryonic Bovine Trachea (EBTr)	Flow Labs	MEM (Earle's) 10% NBCS 1% NEAA	1:2	Fibroblast	37°C	55+
Bovine Kidney (MDBK)	Flow Labs	MEM (Earle's) 10% FCS 1% NEAA	1:10	Fibroblast	37°C	125+
Canine Kidney (MDCK)	-	DMEM (Earle's) 10% NBCS	1:10	Epithelial	37°C	-
Mammalian (Flow) Intestine (1100)	Flow Labs	BME (Earle's) 10% FCS 1% NEAA	1:3	Fibroblast	37°C	-
Human Cervical Carcinoma (HeLa)	May and Baker (Dagenham)	BME (Hank's) 10% NBCS	1:6/1:10	Fibroblast	37°C	300+
McCoy (irradiated)	Institute of Opthalmology	BME (Hank's) 10% NBCS	1:3	Epithelial	37°C	-
Bladder Epithelial	May and Baker (Dagenham)	BME (Hank's) 10% NBCS	1:6	Epithelial	37°C	45+

a   MEM - Minimal Essential Medium
   DMEM - Minimal Essential Medium (Dulbecco's Modification)
    BME - Basal Medium Eagle
   NBCS - Newborn Calf Serum
    FCS - Foetal Calf Serum
   NEAA - Non-Essential Amino Acids

and the pellet washed once or twice in M199, and finally resuspended in M199 (Earle's Salts) and the suspension of neutrophils (2×10^5/ml) was added to 32mm coverslips, for study by reflection interference microscopy. Sporozoites of E. tenella were added approximately 30 minutes afterwards.

2.4.4 Irradiation of chick kidney cells (CK cells)

CK cells were irradiated with gamma rays from a Cobalt-60 source at 5400 rads over a 12 minute period. Fresh growth medium was added and cultures were left at 41°C for 48 hours before being used in experiments.

2.4.5 Infection of cells with parasites

The growth medium was removed from the cells and replaced with the inoculation medium (unsupplemented HBSS). This contained sporozoites at the concentration of 2×10^5 for 13 and 16mm coverslips, 1.5×10^6 for 32mm coverslips and 2×10^6 for 35mm tissue culture dishes.

For experiments to test the inhibitory action of various compounds against invasion, coverslips were washed three times in unsupplemented HBSS to remove all traces of serum, placed in new wells and then the inoculation medium containing sporozoites and compound to be tested was added. After 24 hours the inoculation medium was removed and replaced with M199 supplemented with 1% heat-inactivated NBCS, 0.25% of a 10%LH solution, 4% of a 4% solution of yeast _extract_ in HBSS, and l-glutamine and penicillin/streptomycin solutions at the concentrations described in Section 2.4.2i.

2.4.6 Growth of cells in coculture

12-24 hours before the beginning of coculture, the group to be parasitised was inoculated with sporozoites as described in 2.4.5; the controls were given the same medium without sporozoites. At the beginning of coculture, parasitised cultures were washed vigorously in three changes of unsupplemented medium to remove any free sporozoites and these coverslips were then placed on to silicone grease blobs on the bottom of the Petri dishes, non-parasitised cultures were treated in a similar manner. Monoculture dishes contained 4 coverslips all of similar cell types and coculture dishes contained 2 coverslips of each cell type. The silicone grease ensured that over the 5 day period of incubation the coverslips remained physically separated. Examination of stained cultures verified that the non-parasitised cultures from coculture dishes did not contain any developmental stages of the parasite.

2.5 Invasion of sporozoites into cells

2.5.1 Treatment of Serum

2.5.1i Heat-inactivated serum

Serum was inactivated by incubating it in a water bath at 56°C for 30 minutes.

2.5.1ii Acid-treated serum

1N HCl was added until the serum was at pH3.0. The serum was then left for 15-20 minutes. Following this incubation time an equivalent amount of 1N NaOH was added to return the pH to its original value, the serum was then filtered to resterilise. A dilution factor was taken into account when using this serum.

2.5.2 <u>Chemical Compounds</u>

The chemicals used are listed in Table 2.2.

2.6 <u>Staining techniques and examination of coverslips</u>

Coverslips were removed from culture, air dried, fixed for at least 2 minutes in methanol and stained in a 20% Giemsa solution in PBS for 40 minutes, rinsed in tap water, air dried and mounted in green euparal.

When comparing the effects of the various media on parasite development, the coverslips were examined with a x100 objective lens. Beginning at the top, the coverslips were scanned from edge to edge, and the parasites within the field of view were counted. After the completion of one scan, the coverslip was moved up for a distance equivalent to the width of 4 fields of view, and another scan was made across the width of the coverslip. When the opposite edge was reached, the coverslip was again moved up for a distance equivalent to 4 fields of view and another scan made.

This procedure was repeated until the bottom of the coverslip was reached. For all other experiments, five or ten separate groups of 100 cells were counted for the presence of parasitic stages.

In experiments comparing the growth of the various strains of E. tenella, allowances were made for the degree of confluency of the cell layer, so that the values between which comparisons were made, represented the number of parasites present in a hypothetical 100% confluent culture.

The sizes of the individual stages of the intracellular parasites were measured from a micrometer eye piece.

Table 2.2 Chemicals used in the inhibition experiments on

sporozoite invasion into CK cells

Name	Type	Company
Bovine Serum Albumin (BSA)	Fraction V Powder	Sigma A 4503
Hyaluronidase	Type 1-S	Sigma H 3506
Ovomucoid	Type 111-O	Sigma T 2011
Soybean trypsin inhibitor (STI)	Type 1-S	Sigma T 9003
p-Tosyl-1-arginine methyl ester HCl (TAME)	-	Sigma T 4626
N-α-p-Tosyl-1-lysine chloromethyl ketone HCl (TLCK)	-	Sigma T 7254

2.7 Radioactive compounds and measurement of their incorporation into cells

2.7.1 Radioactive compounds

Radiochemicals were obtained from the Radiochemical Centre, Amersham, U.K. and these are shown in Table 2.3, with their specific activities and the catalogue code numbers. Stock compounds were stored at 4°C.

Table 2.3 - Radioactive Compounds

Compound	Specific activity	Catalogue No.
6-^{3}H Thymidine	26 Ci/mMol	TRK 61
L-1-^{3}H Fucose	6.7 Ci/mMol	TRA 366

2.7.2 Preparation of samples for autoradiography

2.7.2i Growth and pulsing of cells

Cells were seeded on to glass coverslips in the manner described previously (2.4.2). The growth medium was removed and HBSS containing ^{3}H-thymidine added; cultures were incubated for the times indicated in the individual experiments. Following the incubation period the coverslip cultures were removed, washed twice in PBS and fixed in fresh methanol:acetic acid (3:1) for 5 minutes. This was removed and replaced with fresh fixative in which the cells were left for at least 20 minutes. The coverslips were removed, washed in running tap water overnight, air dried and attached, cell side upwards, to microscope slides with Depex mountant (Gurr, Ltd.) and processed for autoradiography.

2.7.2ii Coating and developing autoradiographs

Microscope slides, with the coverslips attached, were coated with a layer of gelatine by dipping them in a solution of 1% gelatin and 0.1% chrome alum. This was allowed to dry and then the slides were coated with a uniform layer of L4 nuclear emulsion (Ilford) diluted 1:1 with distilled water and warmed to 43°C. The emulsion was allowed to dry by placing the slides on ceramic tiles held over ice. Dry slides were placed in light tight boxes containing silica gel, sealed with insulating tape, wrapped in black polythene bags and kept at 4°C for 6 days. Trial development of test slides was always performed to prevent under-exposure.

Autoradiographs were developed for $7\frac{1}{2}$ minutes at 23°C in Phen-X (Kodak) diluted 1:1 with distilled water, washed briefly in a 1% acetic acid stop bath, fixed for 4 minutes in Amfix (Ilford) and washed for at least 20 minutes in running tap water. The preparations were then stained in a 20% Giemsa solution for 40 minutes and air dried.

2.7.3 Preparation of samples for scintillation counting

The growth medium was removed and 1 ml of HBSS with 5-10% NBCS, 25% L.H. (10% solution in HBSS) and L-1-^{3}H-fucose, at known concentrations between 2 and 5µCi/ml, was added to each well. The cultures were incubated at 41°C for 48 hours after which non-labelled fucose at 1000 times the concentration of the labelled compound was added to each well in 1 ml of HBSS. This procedure displaced most unincorporated labelled fucose from the cells. The cultures were incubated for a further 24 hours. Following this 72 hour incubation coverslips were removed, washed three times in unsupplemented medium and placed in fresh wells with the addition of HBSS (with or without sporozoites) supplemented with 1% heat-inactivated NBCS. At various

intervals following inoculation of cultures the coverslips were

removed, fixed in two changes of fresh methanol/acetic acid (3:1) for

5 minutes each, air dried and then treated with three changes of 10%

trichloroacetic acid (T.C.A.) at 4°C for 5 minutes. Following this

precipitation treatment they were washed with running tap water

over-night, air dried, and placed in scintillation vials. 0.5 mls of

Hyamine-IOX was added to each vial and these were kept at 40°C for

24 hours to digest the cellular matter. Acetic acid was added to

neutralise the solution followed by 10 mls of modified Bray's Scintillant

(Galley D.J. and Foerster L.A. 1976). The vials were kept in the dark

for 24 hours, a time which preliminary experiments had shown to be

sufficient to allow chemiluminescence to decay. The radioactive

content was measured with a Beckman LS-250 Scintillation Counter using

the pre-set ^{3}H channel. At the time the coverslips were removed and

processed, the corresponding supernatants were removed and an equal

volume of cold 10% T.C.A. added. These precipitates were left at 4°C

for at least 2 hours and then collected on glass fibre discs (GF/C;

Whatman). The precipitate was washed three times with 5% T.C.A., twice

with ethanol:ether (3:1), and once with ether. The dried discs were

placed in scintillation vials and 10 mls of modified Bray's Scintillant

added. They were then treated as described for the coverslip

preparations.

2.8 Scanning Electron Microscopy

Cultures for scanning electron microscopy were grown and inoculated

in the standard manner (2.4.2 and 2.4.5). All solutions used for

processing were filtered. The coverslips were removed, washed briefly

in 0.1M Sørensen's Phosphate Buffer and fixed at 4°C for 2 hours in 2.4%

glutaraldehyde in HBSS (unsupplemented). After fixation they were

washed in 0.15M Sørensen's Phosphate Buffer and then dehydrated

through a graded series of acetones 10,20,35,50,70,80,90 (twice), 95

and 100% (twice). They were critical point dried in a Polaron 30000

critical point drying apparatus, and mounted on stainless steel stubs

with double-sided sellotape. The stubs were painted around the edges

with silver dag (to reduce the charging of the surface in the microscope)

and then coated with a layer of gold in a sputter-coater in an

atmosphere of argon for 2 minutes. Preparations were examined at an

angle of 45° in a Cambridge Stereoscan Microscope.

2.9 Concanavalin A (Con A) agglutination tests

Cultures were washed twice with PBS without Ca and Mg and then

incubated in PBS without Ca and Mg at 37°C until the cells began to

round up (generally 20-30 minutes). The incubation medium was poured off

and the cells were removed by directing 5 mls of ice cold PBS (complete)

over the cells from a pipette. Any clumps of cells were dispersed by

gently passing up and down a 10 mls syringe fitted with a 19g needle.

The resulting single cell suspensions were collected on ice and spun down

at 800 r.p.m. at 4°C for 10 minutes and then resuspended at a

concentration of between 1-2 x 10^6 cells in 2 mls of HBSS containing

DNAase at 1mg/ml (Sigma Chemicals DN-25) and with or without Concanavalin

A (Con A, Sigma Chemicals, Grade IV) at 0.1 - 100μg/ml. The cells were

then placed in a water bath at 31°C and shaken at 75 r.p.m. for 30 minutes.

Solutions were removed and scored for agglutination; all assessment of

agglutination was "done blind". To assess the ability of fixed cells to

agglutinate single cell suspensions were held at 4°C in a 2%

glutaraldehyde solution in HBSS for 30 minutes. This fixative was removed,

the cells washed in unsupplemented HBSS, resuspended in the

agglutination medium and treated as above. Agglutination was generally

scored on a + to +++++ scale. However in the experiment where fixed

cells were compared with normal cells agglutination was scored on the

percentage of single cells present, as background aggregation was high.

2.10 Reflection Interference Microscopy

This method was used to investigate the nature of adhesions

present between the CK cells and their substrate (Curtis 1964,1973).

Normal cells were compared with parasitised cells. Cultures were

established and inoculated as described earlier (Section 2.4.2 and 2.4.5)

and examined with the reflection interference microscope as described

by King et al (1979).

SECTION 3: RESULTS

3.1 Development of Eimeria in tissue culture

3.1.1 Development of E. tenella in various media systems

The growth of E. tenella in CK cells was tested under a variety

of conditions in which the medium and medium supplements were used in

various combinations for inoculation and maintenance. All cultures

were initially grown in HBSS with 10% NBCS and 2.5% of a 10% lactalbumin

hydrolysate (LH) solution. All maintenance media contained 0.25% of

this LH solution.

Table 3.1.1 lists the various combinations of inoculation media

and maintenance media which were used. The percentage of gametocytes

(mature and immature) and the total number of intracellular stages

found on the 7th day p.i. is illustrated in Fig. 3.1.1. The condition

of the cells at this time, scored on a scale +(poor growth) to +++++(good

growth) is also given in Fig. 3.1.1.

With 4% yeast in the inoculation medium (Systems F and G) the

yield of all intracellular stages on day 7 p.i. was low. With 2% yeast

in the inoculation medium slightly better results were obtained

(Systems A,B and C). However, the best gametocyte development was seen

in systems where yeast was omitted from the inoculation medium (Systems

D and E). Without yeast not only was the percentage of gametocytes high

but also the actual number of gametocytes was greatest.

HBSS, supplemented with 10% NBCS was a satisfactory medium for CK

cells. However, when supplemented with 1% NBCS it was not a good growth

medium for E. tenella within these cells. MEM + 1% NBCS gave a good

production of gametocytes (Fig. 3.1.1; D5). However, it was not a

reliable medium for parasite or cell growth and was not chosen as a

maintenance medium. M199 + 1% NBCS gave good results in almost all cases

Legend for Table 3.1.1.

NBCS - New Born Calf Serum

2%/4% Yeast - 2%/4% of a 4% solution of Yeast in HBSS

M199 - Medium 199 (with Hank's Salts)

BME - Basal Medium Eagle's (with Hank's Salts)

HBSS - Hank's Balanced Salt Solution

SF - Serumless Medium (Neuman & Tytell)(Gibco-Biocult.163)

L15 - Leibovitz Medium L-15

MEM - Minimal Essential Medium.

Table 3.1.1. Media systems used to inoculate and maintain CK cultures

parasitised with E. tenella

SYSTEM	MEDIUM FOR INOCULATION	MEDIUM AFTER INOCULATION
A1	M199 + 2% Yeast	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
B1	BME + 2% Yeast	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
C1	HBSS + 2% Yeast	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
5		MEM + 1% NBCS
D1	BME + 1% NBCS	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
5		MEM + 1% NBCS
E1	HBSS + 1% NBCS	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
5		MEM + 1% NBCS
6		M199 + 1% NBCS + 4% Yeast
F1	BME + 4% Yeast	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
5		MEM + 1% NBCS
6		M199 + 1% NBCS + 4% Yeast
G1	M199 + 4% Yeast	BME + 1% NBCS
2		M199 + 1% NBCS
3		HBSS + 1% NBCS
4		SF
5		MEM + 1% NBCS
6		M199 + 1% NBCS + 4% Yeast
7		L15
8		BME w/o phenol red + 1% NBCS

Fig. 3.1.1. The growth of E.tenella at 7 days p.i. under a variety of conditions
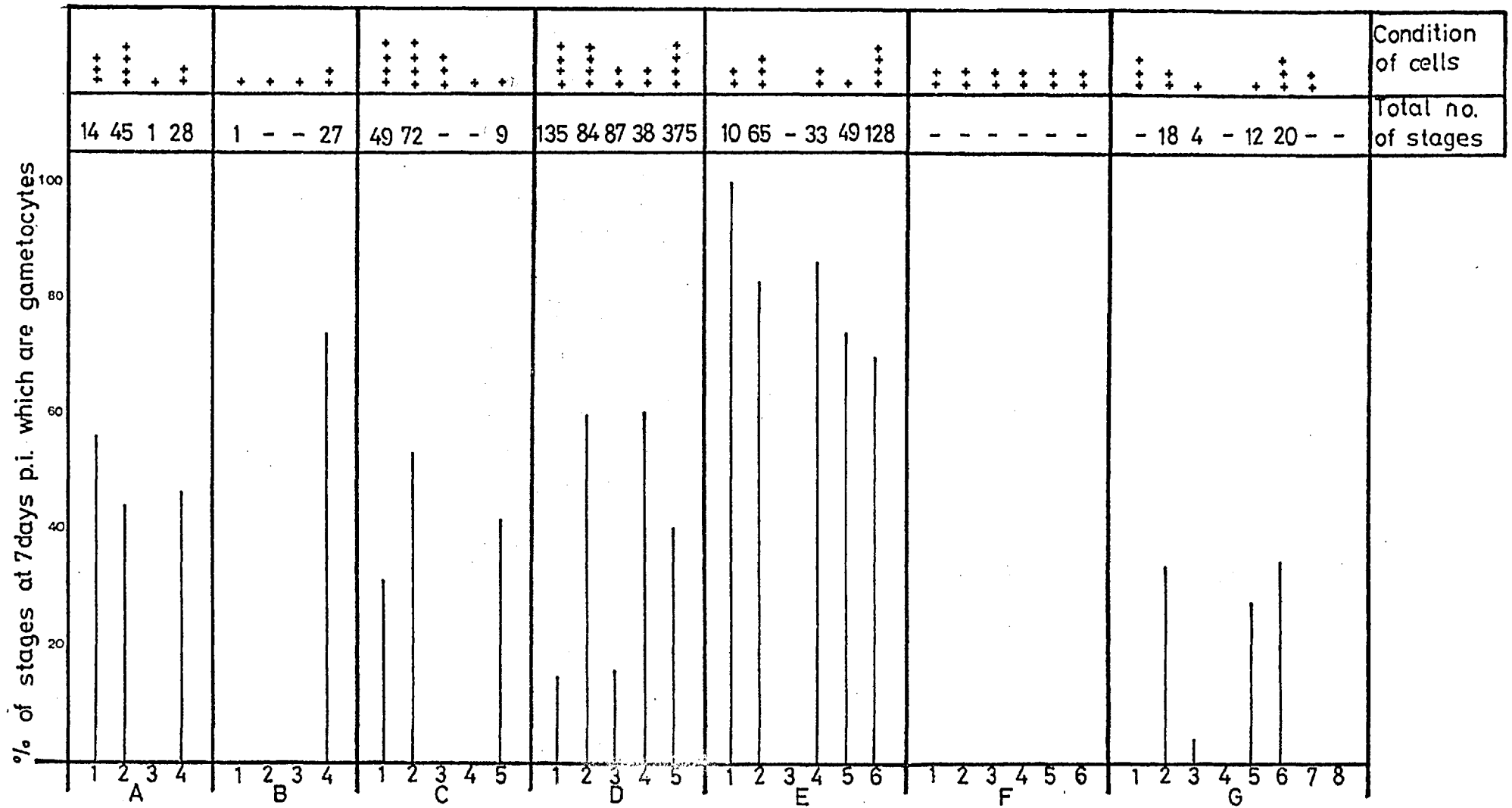
and was also a good medium for maintenance of cells up to day 7 p.i.
                                                                  extract
Also the percentage of gametocytes was high. When 4% yeast was added
                                                      the   growth
to M199 it supported even better development of parasites and cell (see

results of E2 and E6). In all experiments HBSS with 10% NBCS was

selected as the growth medium for cells, HBSS (without supplements)

was used as the inoculation medium and M199 with 1% NBCS and 4% yeast extract

(of a 4% solution in HBSS) was used for the maintenance of cultures.

3.1.2 Development of E. tenella, from a sporozoite inoculum, in non-host cells

A variety of non-host cells were used to test for the development of E. tenella. Although normally grown at 37°C these cell lines were grown at the higher temperature of 41°C for these experiments. The extent of development is given in Table 3.1.2. The sporozoites were capable of invading several of the cell types on to which they were inoculated. No results could be obtained for Flow 11,000, MDCK and EBTr cells because these cells did not survive the first 24 hours at 41°C, which was when the first preparations were fixed.

3.1.2i Bovine Kidney Cells (MDBK)

These cells grew very well at 41°C producing a contact-inhibited, epithelial monolayer. They maintained a reasonable appearance, even after six days of growth at this temperature (Fig. 3.1.2). Despite this, the parasites did not show any increase in size nor change of shape which would have indicated development beyond the intracellular sporozoite stage (Fig. 3.1.3).

3.1.2ii Human Cervical Carcinoma Cells (HeLa)

Table 3.1.3 shows the results of counting the number of cells parasitised by E.tenella at 24 hours post-inoculation (p.i.). There were three groups distinguished by the temperatures of growth pre- and post-inoculation.

Table 3.1.3 The invasion of E. tenella sporozoites into HeLa cells, growing at various temperatures

Group	Temperature pre-inoculation	Temperature post-inoculation	Number of cells parasitised
A	41°C	41°C	8.3 ± 1.77
B	37°C	41°C	33 ± 4.90
C	37°C	37°C	5.0 ± 2.05

Table 3.1.2 Growth of E. tenella from sporozoites inoculated on to cells from non-host tissue

Cell	Characteristics	Invasion by sporozoites	Other development	Conditions of cells at 3 days p.i.
Bovine Kidney (MDBK)	Cell Line	✓	-	Good
Bovine Trachea (EBTr)	Cell Line	-	-	Dead
Human Intestine (Flow 11000)	Cell Line	-	-	Dead
Human Cervical Carcinoma (HeLa)	Cell Line	✓	-	Deteriorating
Canine Kidney (MDCK)	Cell Line	-	-	Dead
Bladder Epithelial	Cell Line	✓	-	Dead
Human Synovial fluid (McCoy)*	Cell Line/Irradiated	✓	-	Deteriorating

* Originally derived from human synovial fluid but later characterised as mouse fibroblasts, suggesting

take-over by contaminant cells
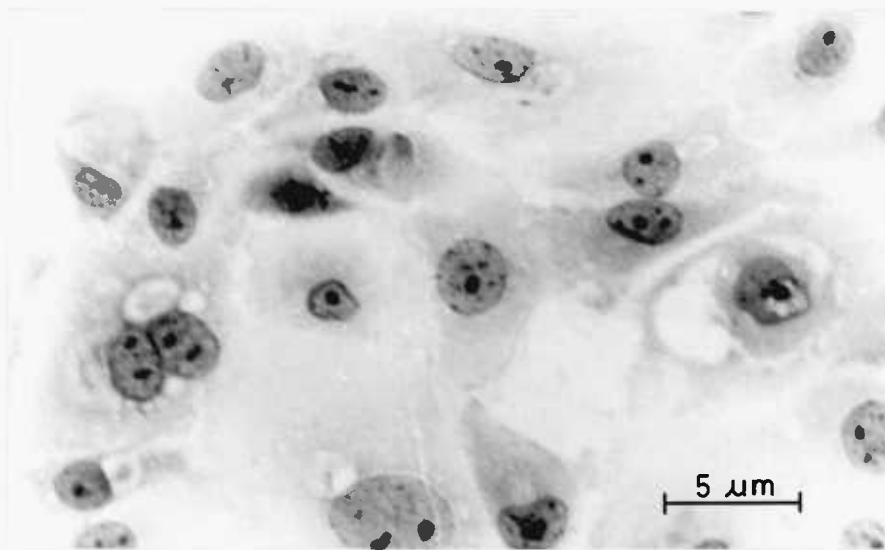
Figure 3.1.2

The appearance of MDBK cells after 6 days growth at 41°C
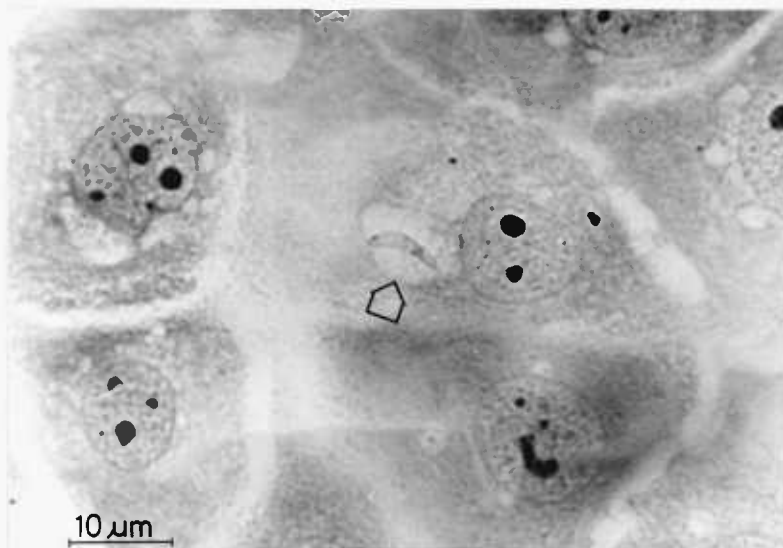
Figure 3.1.3

Intracellular sporozoite in MDBK cells 1 day p.i./41°C

All groups were invaded by sporozoites, with group B apparently providing a more suitable environment as the number of cells parasitised was more than four times as great as in the other two groups. No further development beyond intracellular sporozoites occurred.

After 3 days of growth at 41°C most cells from these cultures had detached, although the few remaining appeared healthy (Fig 3.1.4a). Cultures growing at 37°C throughout the experiment, appeared fairly confluent at 3 days p.i. (Fig. 3.1.4b).

3.1.2iii <u>Bladder Epithelial Cells</u>

A few intracellular sporozoites were observed inside cells which were grown at either 41°C or 37°C p.i. (Fig. 3.1.5). However, deterioration of the cell layer resulted in most of the cells becoming detached within 48-72 hours, and no further development was observed.

3.1.2iv <u>McCoy cells</u>

Cultures did not grow well at 41°C, but sporozoites entered the cells at this temperature and those grown at the normal temperature of 37°C. It was very common to see mass invasion of cells (Fig. 3.1.6). This culture became composed of an increasing number of large cells, containing lobed nuclei as the days post-inoculation increased (Fig. 3.1.7). This phenomenon was a feature of both parasitised and non-parasitised cells.

3.1.3 Development of <u>E. tenella</u>, from a sporozoite inoculum, in host cells

Primary cultures were established from a variety of embryonic and adult chick tissues. Table 3.1.4 shows the extent of development in all types. Except in embryonic and adult kidney and embryonic liver, there was no development beyond the first generation schizogony.

Figure 3.1.4   HeLa cells grown for 3 days at:

10 μm

a) 41°C

10 μm

b) 37°C

Figure 3.1.4   HeLa cells grown for 3 days at:
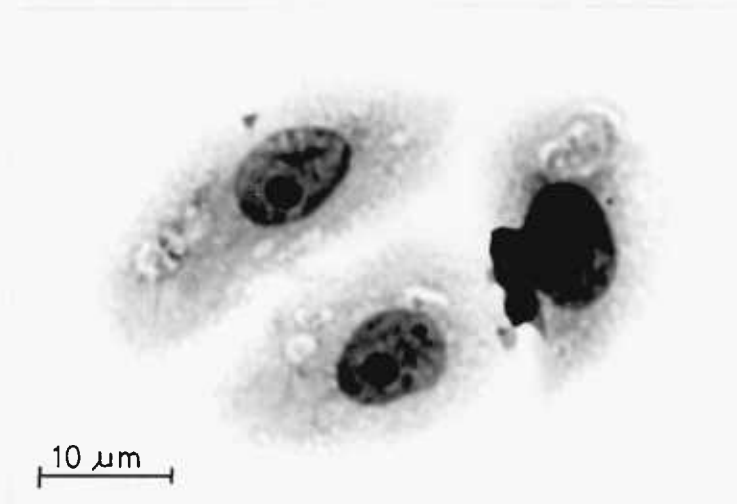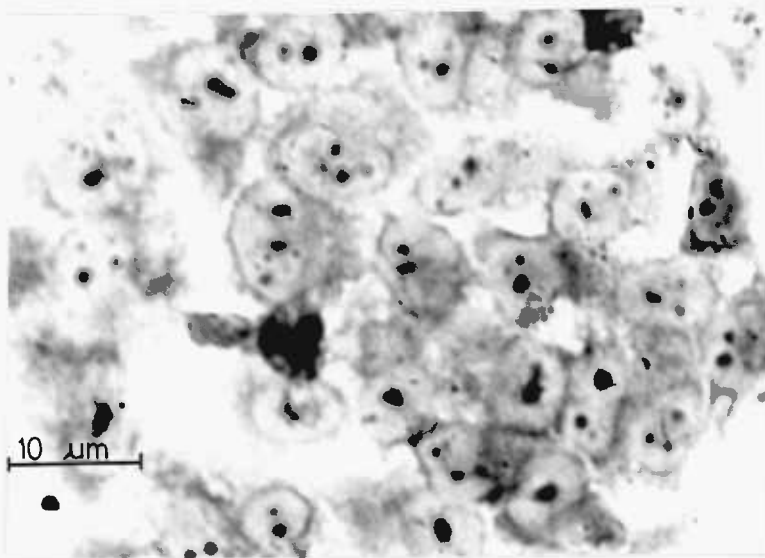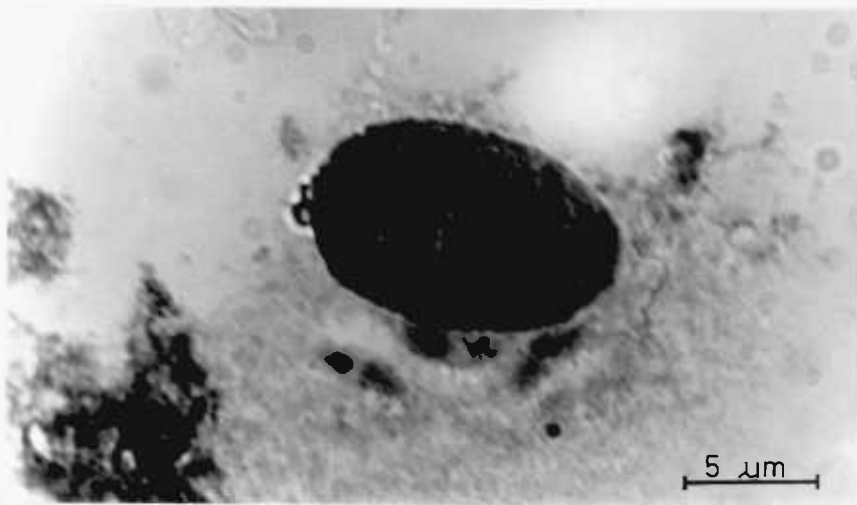
Figure 3.1.5

Bladder epithelial cells containing intracellular sporozoites
2 days p.i./41°C
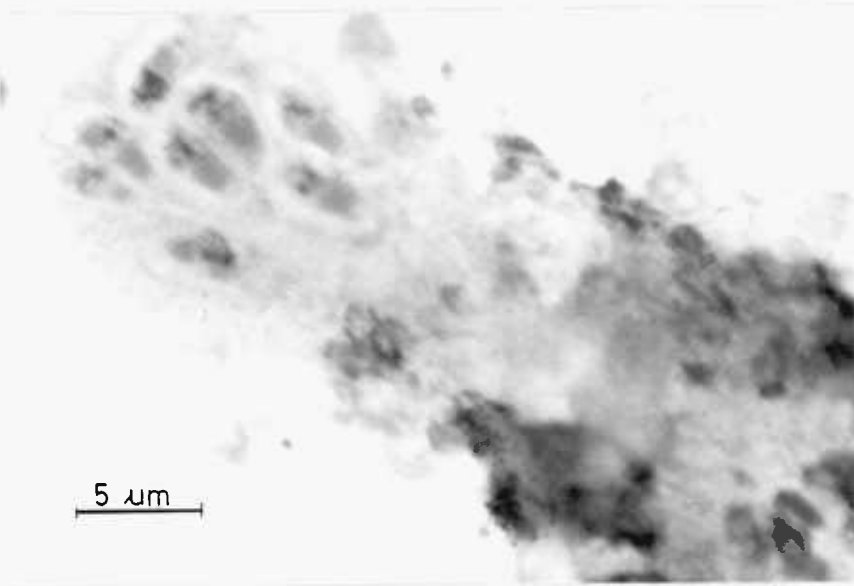
Figure 3.1.6

Multiple invasion of McCoy cells 1 day p.i./41°C
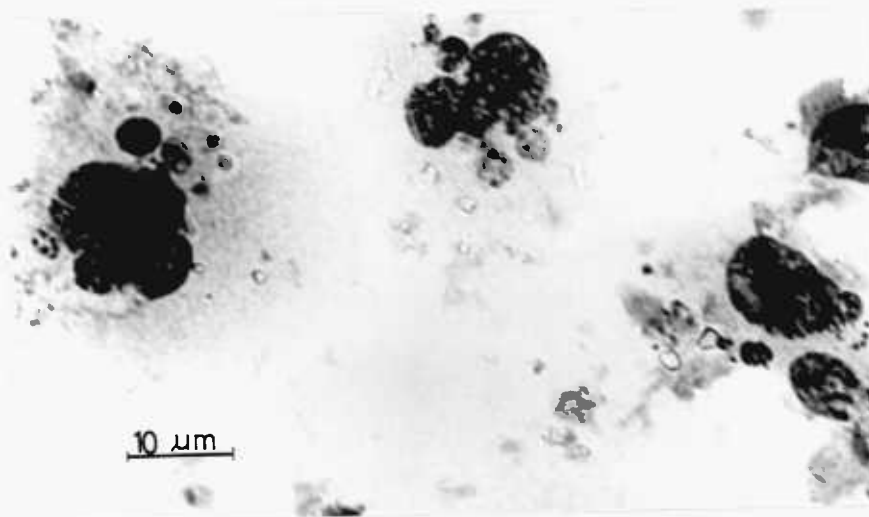
Figure 3.1.7

McCoy cells after 5 days growth at 37°C with many cells having multi-lobed nuclei

Table 3.1.4 Development of E. tenella from sporozoites inoculated on to host cells

Cell Type[a]	Invasion by sporozoites	Most frequent stage reached[b]	Furthest stage reached[b]
Chicken Embryonic Kidney (CEK)	✓	mature gametocytes	oocysts
Chicken Kidney (CK)	✓	mature gametocytes	mature gametocytes
Chicken Embryonic Liver (CEL)	✓	immature schizonts[2]	mature gametocytes
Chicken Embryonic Spleen (CES)	✓	intracellular sporozoite	trophozoite[1]
Chicken Embryonic Lung	✓	trophozoite[1]	immature schizont[1]
Chicken Embryonic Macrophages	✓	intracellular sporozoite	immature schizont[1]
Chicken Macrophages	✓	-	-
Chicken Embryonic Intestine	✓	immature schizont[1]	mature schizont[1]

a - all primary cultures
b - number represents generation

3.1.3i Chick Embryonic Spleen

The sporozoites readily entered these cells but only occasionally developed further. Fig. 3.1.8 illustrates a trophozoite found in a culture of these cells 4 days p.i. The lack of any mature first generation schizonts suggested that this trophozoite was of the first generation and not the second, despite the fact that it was seen 24-48 hours later than expected. Possibly its development had been arrested.

3.1.3ii Chick Embryonic Lung

Many trophozoites were observed in chick embryonic lung cells at 48 hours p.i. Immature first generation schizonts also began appearing at this time but did not develop beyond the six-nucleate stage (Fig. 3.1.9)

3.1.3iii Chick Embryonic Intestine

This tissue supported the development of rare mature first generation schizonts; merozoites of this generation were separated on the fourth day p.i. (Fig. 3.1.10).

3.1.3iv Chick Embryonic Macrophages

Sporozoites readily invaded these cultures, but little further development was observed. In one culture, however, a large immature schizont was found 3 days p.i. (Fig. 3.1.11).

3.1.3v Chick Embryonic Liver

The sporozoites of E. tenella readily invaded these cells and developed to the second generation schizonts. By day 6 p.i. a few immature gametocytes were observed. (Fig. 3.1.12a and b). The presence of groups of trophozoites and a few small schizonts at this time suggested that many second generation merozoites once released had entered cells to develop into a third generation of schizonts rather than into the
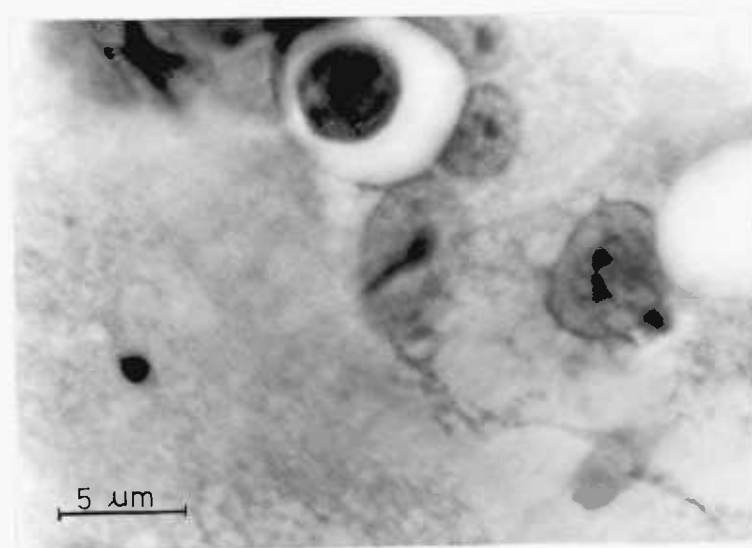
Figure 3.1.8

Trophozoite of E. tenella in chick embryonic spleen cells, 4 days p.i.
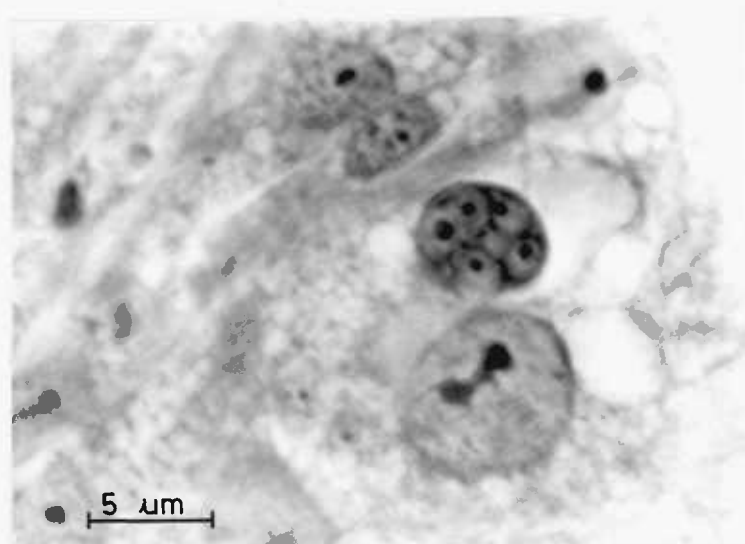
Figure 3.1.9

Immature schizont of E. tenella in chick embryonic lung cells at 2 days p.i.
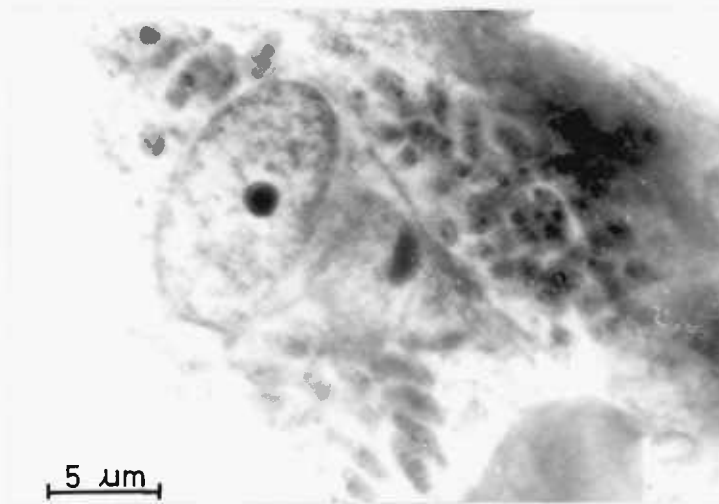
Figure 3.1.10

Mature first generation schizont of <u>E. tenella</u> with separated merozoites
at 4 days p.i. in chick embryonic intestine cells
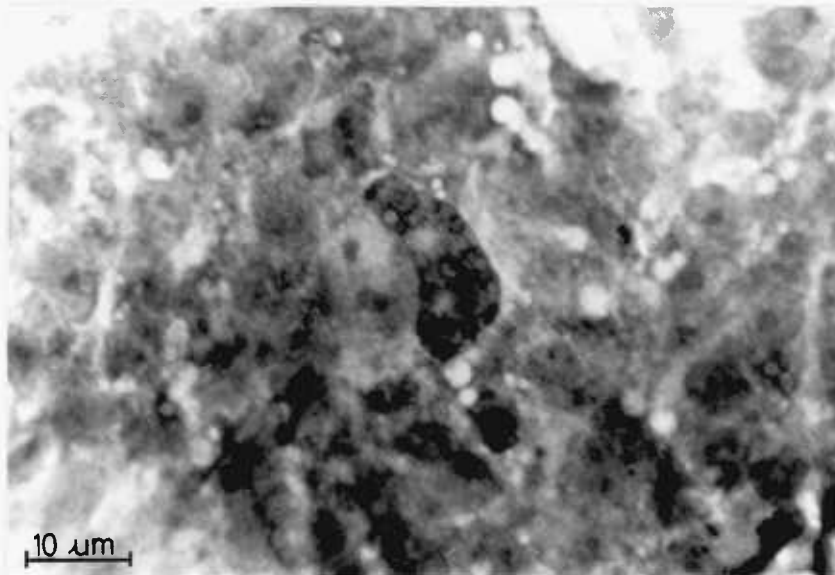
Figure 3.1.11

Immature first generation schizont of <u>E. tenella</u> growing in chick
embryonic macrophages, 3 days p.i.

Figure 3.1.12   Immature gametocytes, 6 days p.i., growing in chick
                embryonic liver cells
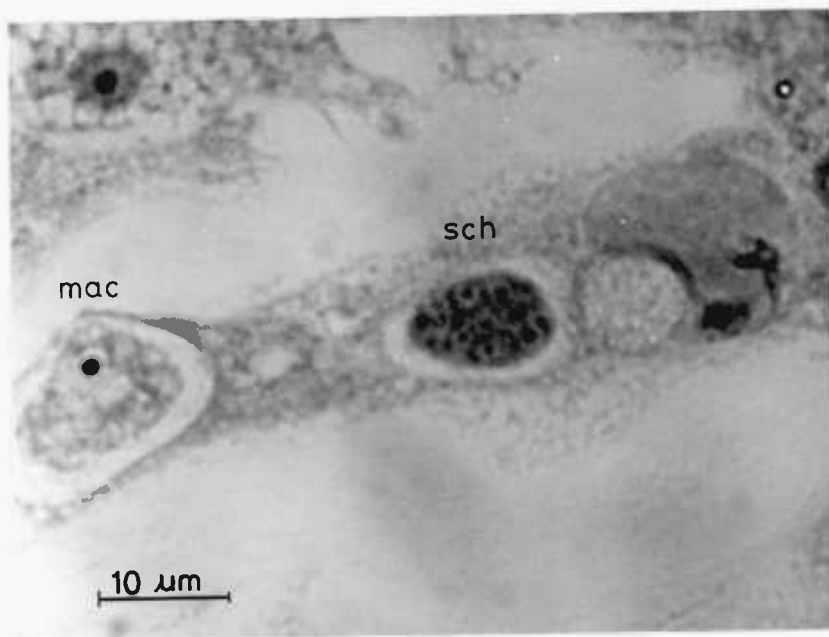
a) Immature macrogamete (mac) with a possible delayed second generation
   schizont (sch).
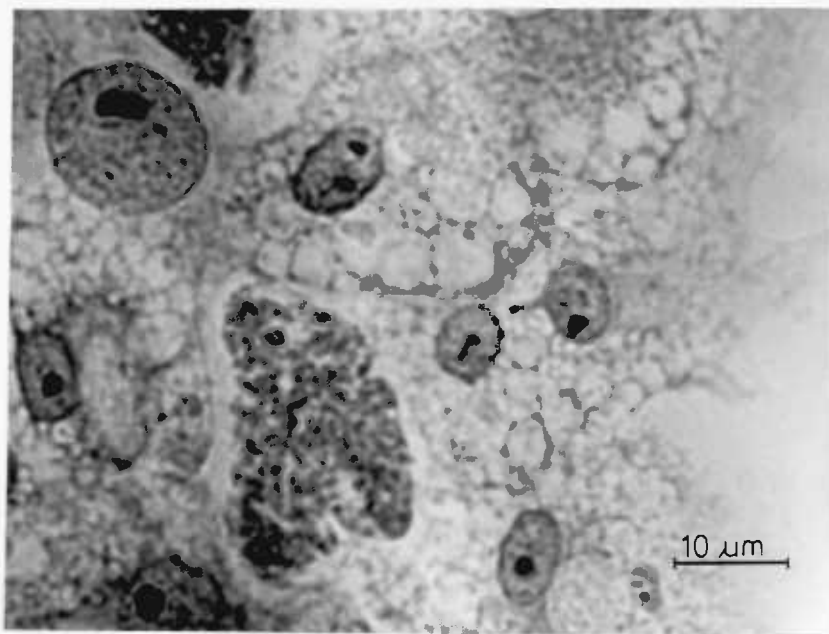
b) Immature microgametocyte

gametocytes. (Figs. 3.1.13 a and b). However, the possibility that they were delayed second generation schizonts cannot be excluded, particularly as the second generation merozoites were sometimes of this size.

3.1.3vi Chick Kidney Cells (Embryonic and Adult)

The sporozoites entered adult and embryonic chick kidney cells which were equally effective in sustaining greater development of the parasite than any other cell type. Measurements of stages of E. tenella growing in CK cells are given in Table 3.1.5. On entering the cells, sporozoites became shorter and broader (Fig.3.1.14a) passing through pear-shaped stages (Fig. 3.1.14b) to produce rounded, uninucleate trophozoites (Fig. 3.1.14c), at 36-48 hours.

After successive nuclear divisions a multinucleate immature schizont was produced. (Fig. 3.1.15). The nuclei of this and the second generation schizonts were vesicular.

Merozoites were separated from the mature schizonts either by budding from the surface so that they were neatly arranged in a rosette formation round a residual mass (Fig. 3.1.16) or more haphazardly (Fig. 3.1.17). Trophozoites, which had formed after re-invasion of cells by first generation merozoites, were present alongside mature schizonts which had not released their merozoites (Fig. 3.1.16, arrows). Occasionally cells were found containing 2-3 schizonts at different stages of development. Fig. 3.1.18 shows one cell containing a young schizont (centre), a mature multinucleate schizont (top) and one which had separated the merozoites (bottom).

Merozoites of this generation averaged 4.7 x 1.8μm, and there were usually between 50-100 merozoites per schizont. Due to the local release

Figure 3.1.13 Third generation schizonts, 6 days p.i., growing in chick embryonic liver cells

a) Immature schizont

Figure 3.1.13 Third generation schizonts, 6 days p.i., growing in chick embryonic liver cells
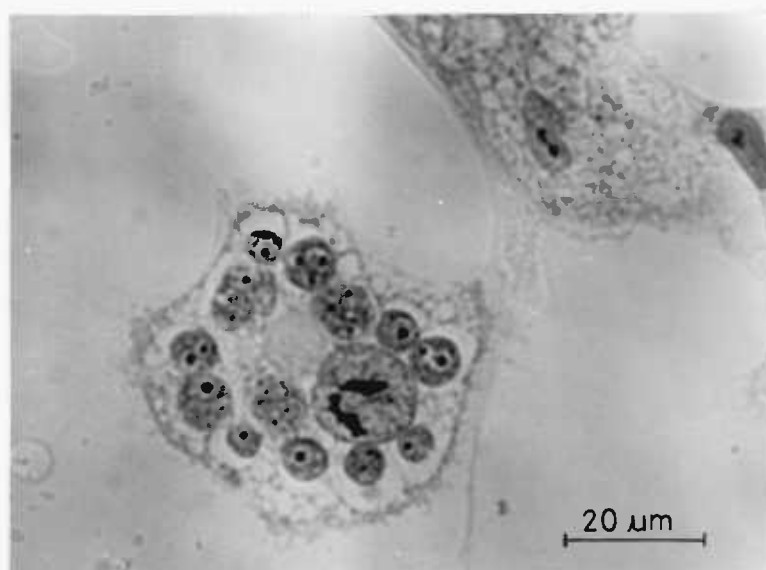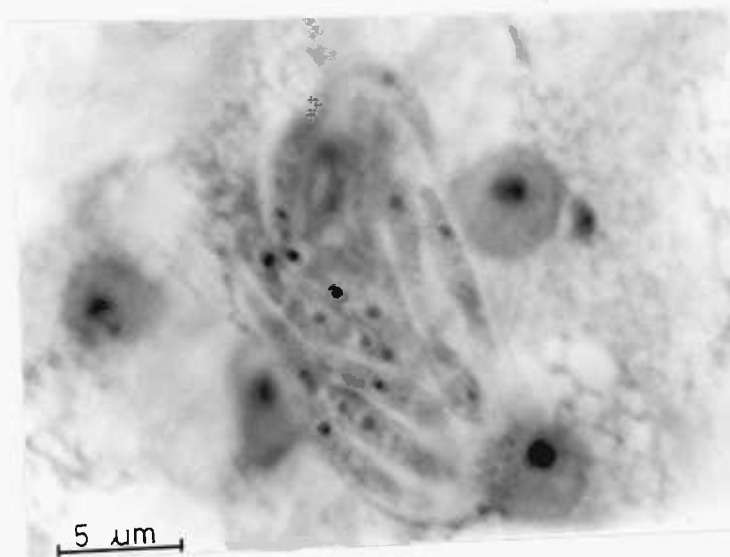
b) Mature schizont

Table 3.1.5 Measurements of intracellular stages of <u>E. tenella</u> in CK cells

Stage[a]	No. Sampled	Days p.i.	Size[b] Mean.	Range
Sporozoite	20	2	7.25 x 3.2 $\pm$ 0.35 x 0.15	11.8 x 4.7 – 4.7 x 2.4
Trophozoite[1]	20	2	8.10 x 6.5 $\pm$ 0.25 x 0.29	9.4 x 8.3 – 7.1 x 5.9
Immature schizont[1]	20	2	15.7 x 10.9 $\pm$ 1.9 x 4.33	41.4 x 23.6 – 8.3 x 7.1
Trophozoite[1]	13	3	7.7 x 5.9 $\pm$ 0.28 x 0.39	9.4 x 5.9 – 7.1 x 4.7
Immature schizont[1]	18	3	22.2 x 13.69 $\pm$ 2.9 x 1.62	48.4 x 36.6 – 8.3 x 7.1
Mature schizont[1]	10	3	44.0 x 21.9 $\pm$ 3.12 x 2.0	60.2 x 41.3 – 28.3 x 17.7
Merozoites[1]	10	3	4.7 x 1.8 $\pm$ 0.20 x 0.19	5.9 x 2.4 – 3.5 x 1.2
Trophozoites[2]	20	4	6.0 x 5.0 $\pm$ 0.32 x 0.28	9.4 x 8.3 – 4.7 x 3.5
Immature schizont[2]	20	4	13.8 x 10.6 $\pm$ 1.2 x 0.84	30.7 x 24.8 – 7.1 x 5.9
Immature schizont[2]	20	5	15.8 x 11.8 $\pm$ 1.25 x 0.80	28.3 x 17.7 – 7.1 x 5.9
Mature schizont[2]	21	6	35.0 x 25.1 $\pm$ 2.7 x 1.4	59.0 x 41.3 – 18.9 x 17.7
Merozoites[2]	21	6	11.8 x 1.5 $\pm$ 0.39 x 0.10	16.5 x 1.2 – 9.5 x 1.2
Immature Macrogametes	12	7	13.4 x 8.3 $\pm$ 2.23 x 0.88	27.1 x 13.0 – 7.1 x 5.9
Mature Microgametocytes	10	7	43.6 x 25.0 $\pm$ 4.9 x 1.6	70.8 x 28.3 – 24.8 x 16.5
Mature schizonts[3]	6	7	19.7 x 15.3 $\pm$ 2.39 x 1.5	28.3 x 21.2 – 12.9 x 9.4

a = number denotes generation
b = measurements in microns

Figure 3.1.14 The transformation of intracellular sporozoites of
E. tenella to trophozoites
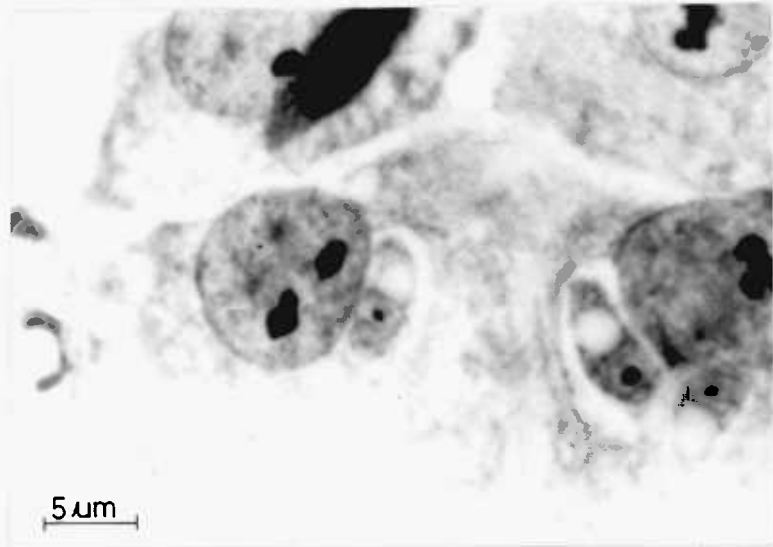
a) Shortening and broadening of sporozoite
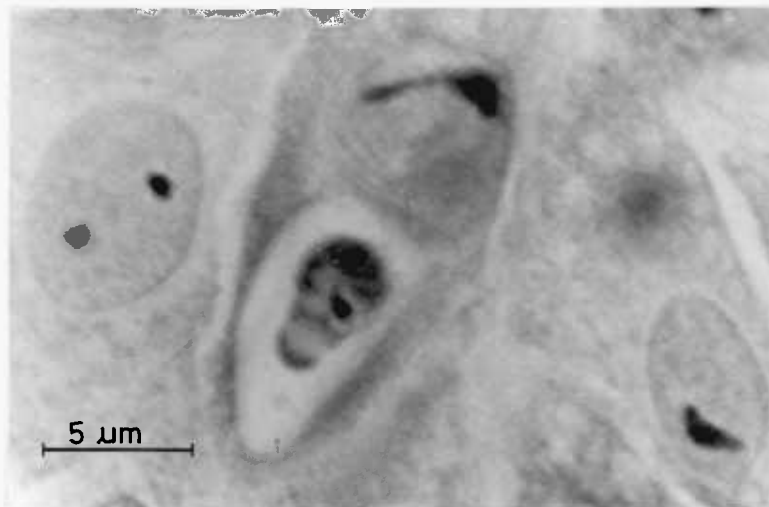
b) Pear-shaped stage

20 μm
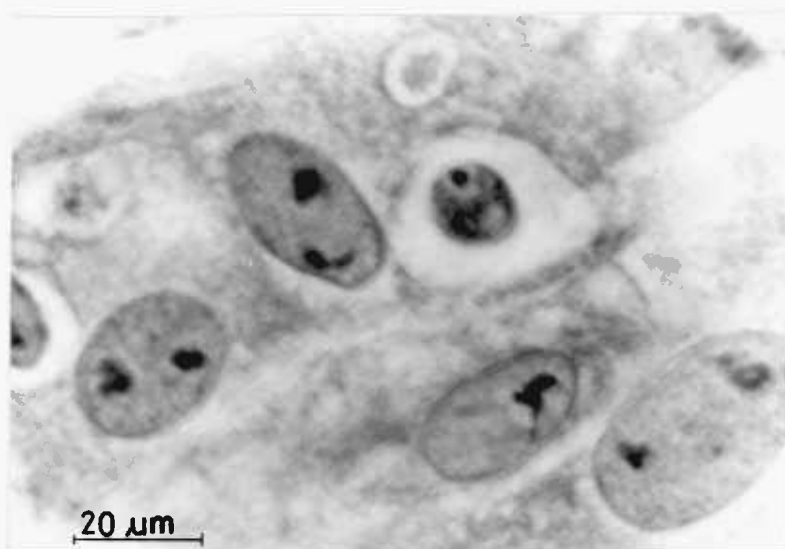
Figure 3.1.14

c) Uninucleate trophozoite
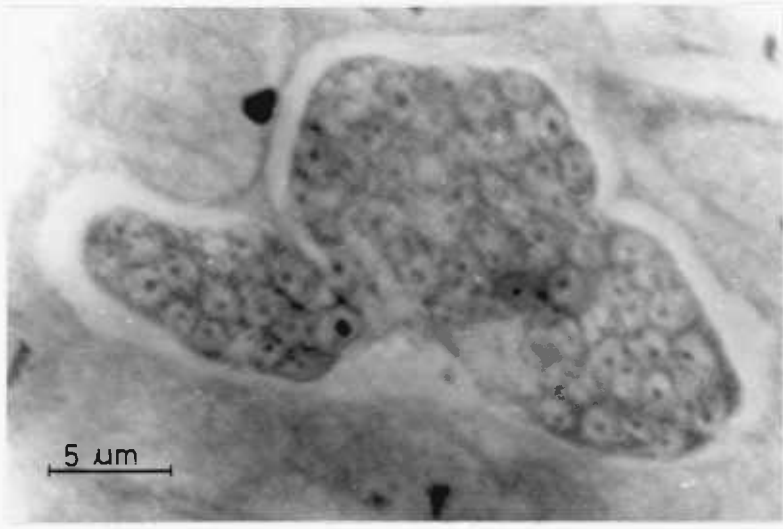
Figure 3.1.15

Immature first generation schizont of E. tenella growing in CK cells
3 days p.i.
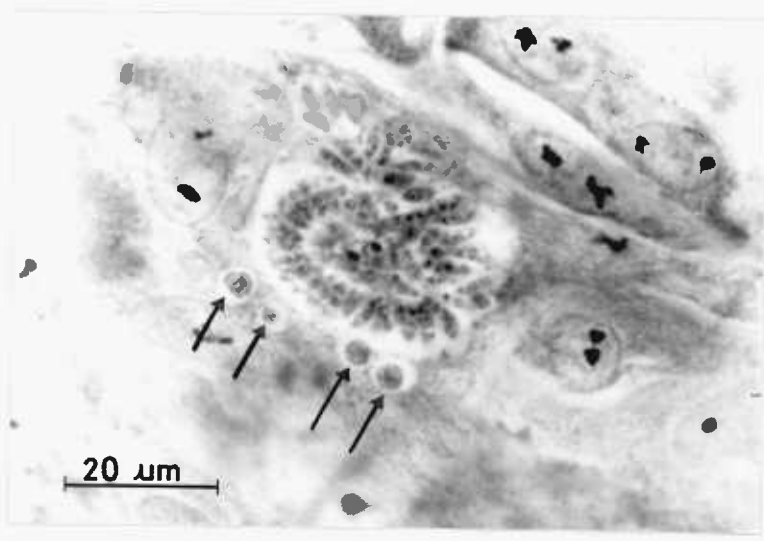
Figure 3.1.16

First generation schizont of E. tenella, growing in CK cells, showing
merozoites arranged in a rosette formation around a residual mass
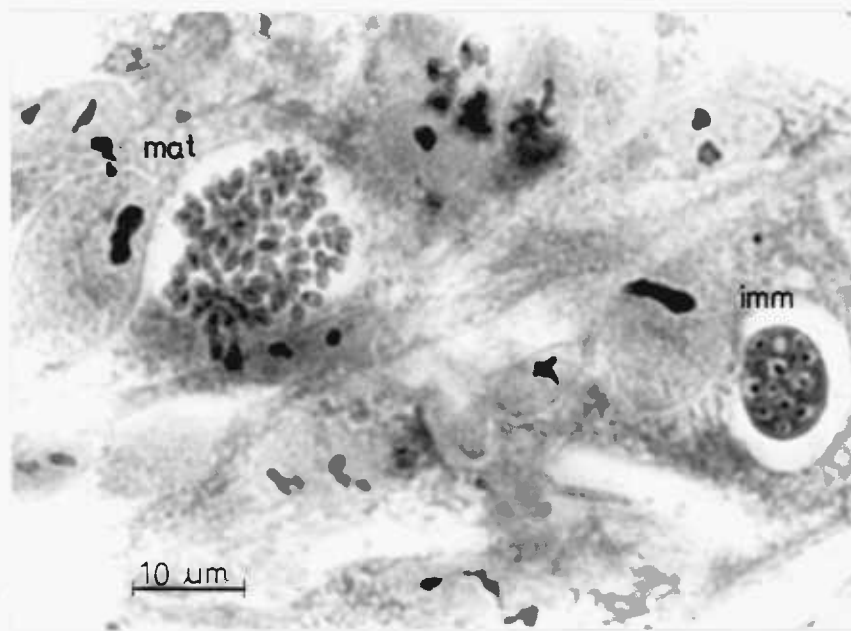
Figure 3.1.17

Mature (mat) first generation schizont of E. tenella 2 days p.i., showing
the merozoites arranged haphazardly; immature (imm) first generation
schizont also present
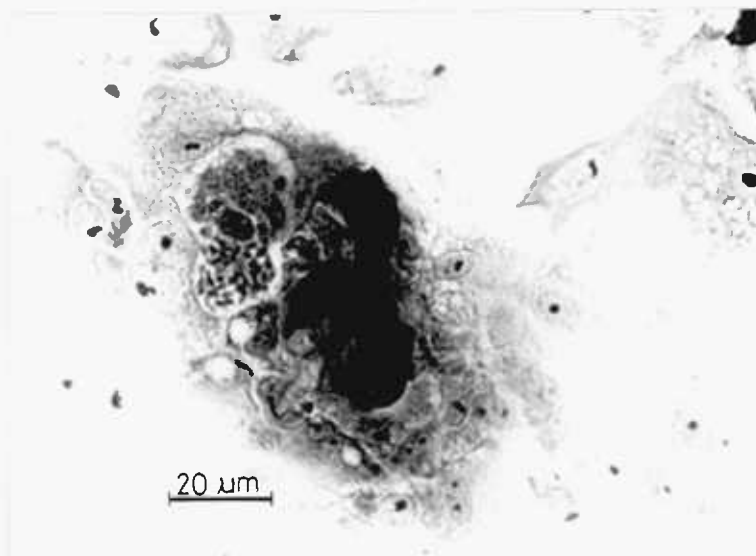
Figure 3.1.18

A CK cell containing three first generation schizonts of E. tenella
all at various stages of development

of large numbers of first generation merozoites, large numbers of

second generation schizonts were found aggregated in high

concentrations in localised areas. There was also a tendency for

the first generation merozoites to invade areas of dense cellular

growth. Such areas were provided by sheets of cells which were

beginning to pile up. These areas were often adjacent to places

where deterioration and detachment of cells was occurring (Fig. 3.1.19).

Schizonts developed from these trophozoites in a similar manner

to the first generation schizonts. Immature schizonts were most

abundant on days 4 and 5 p.i. (Fig. 3.1.20). By day 5 mature schizonts

were common. Clumps of infected cells lay close together and contained

small schizonts, which separated into bundles of 6-15 merozoites

arranged like bunches of bananas (Fig. 3.1.21). Some larger schizonts,

which were usually isolated rather than crowded, had merozoites

arranged neatly around a residual mass (Fig. 3.1.22). Sometimes a few

nuclei remained in the residual mass and were not incorporated into

merozoites (Fig. 3.1.22, arrows). In other schizonts merozoites were

arranged haphazardly, apparently without residual cytoplasm (Fig. 3.1.23).

Some schizonts divided into separate cytoplasmic masses of

varying sizes, containing several nuclei, or into spherical bodies

containing one nucleus (Fig. 3.1.24, a and b). These were thought to

be blastophores although their growth and further development into

merozoites was not observed.

The largest, single schizonts observed at 5 days p.i. contained

100 or more merozoites. Occasionally very large schizonts were found

(Fig. 3.1.25) but these probably represented several schizonts which

merged after breakdown of adjacent parasitophorous vacuoles.
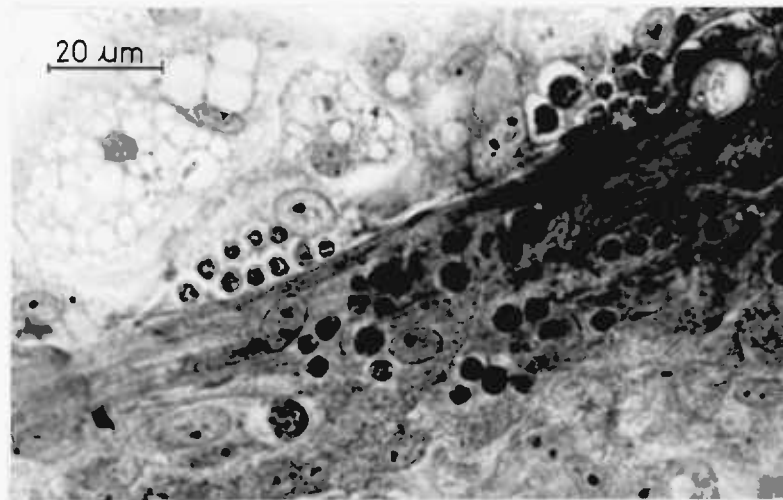
Figure 3.1.19

Second generation trophozoites of E. tenella preferentially invade and develop in areas where the CK cells are growing more than one cell deep
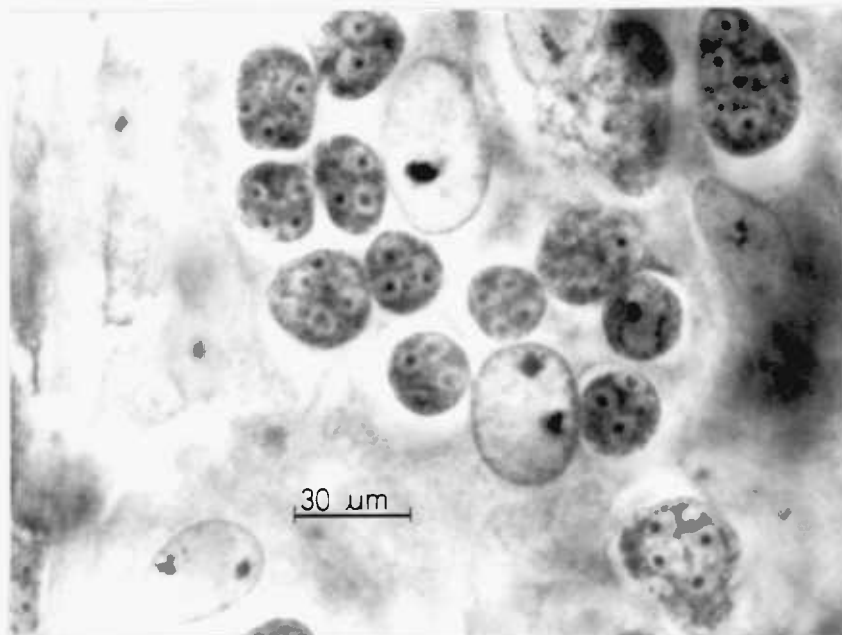
Figure 3.1.20

Immature second generation schizonts of E. tenella, 4 days p.i., growing in CK cells
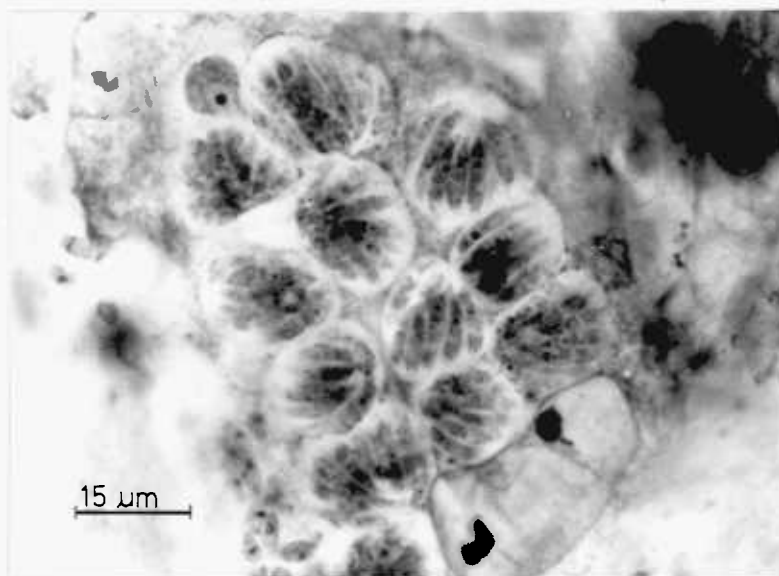
Figure 3.1.21

The local invasion of CK cells by first generation merozoites produces small mature second generation schizonts, developing in closely spaced groups
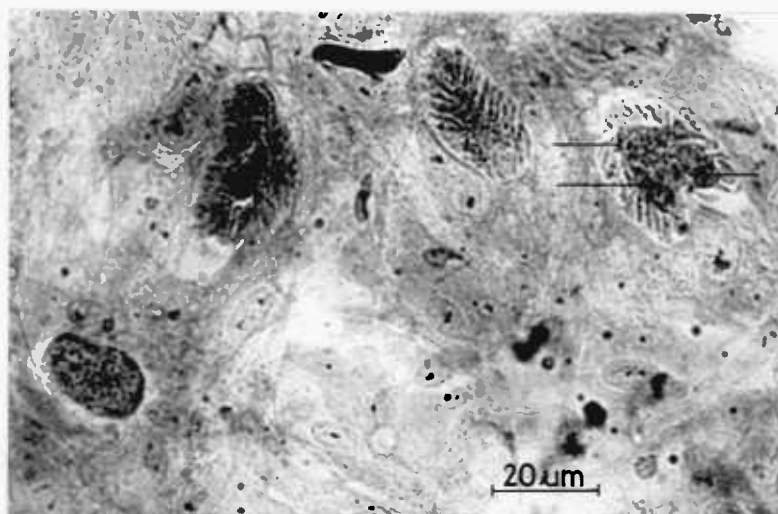
Figure 3.1.22

Mature second generation schizont with rosette formation of merozoites, 6 days p.i. (unincorporated nuclei are marked by arrows).
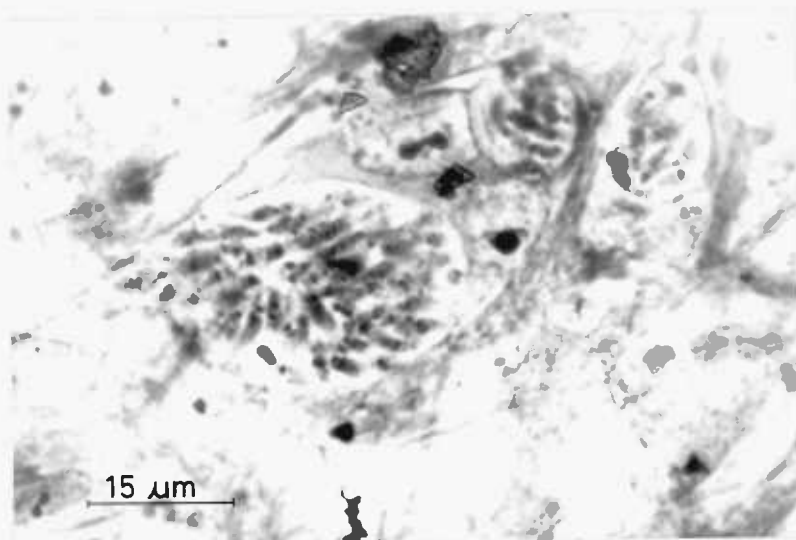
Figure 3.1.23

Mature second generation schizont of E. tenella with haphazard
arrangement of merozoites, 7 days p.i.

Figure 3.1.24 Mature second generation schizonts of E. tenella with the cytoplasm divided into several separate groups of nuclei (blastophore schizont)

15 μm

a)

5 μm
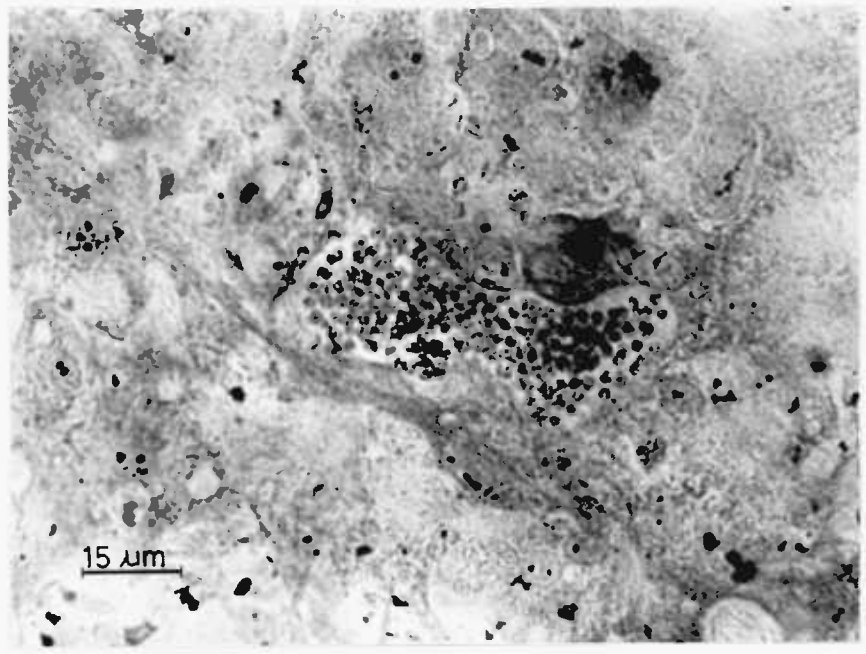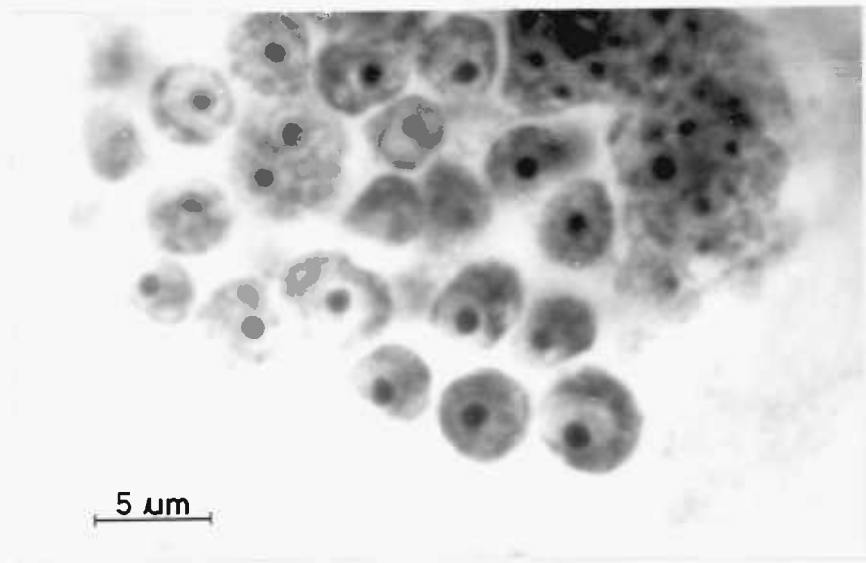
b)
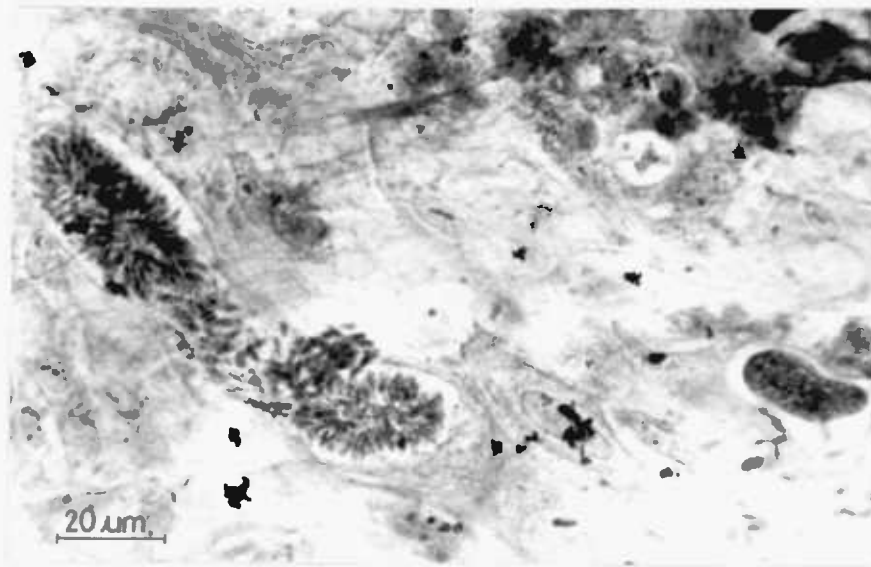
Figure 3.1.25

Mature second generation schizont of E. tenella - fusion of two parasitophorous vacuoles has produced a large schizont
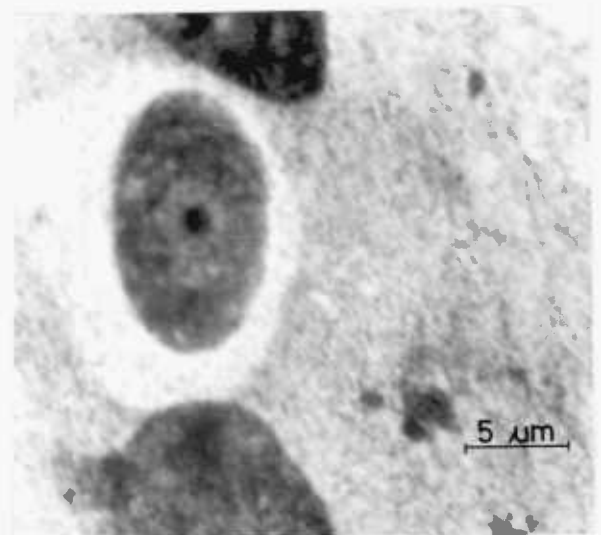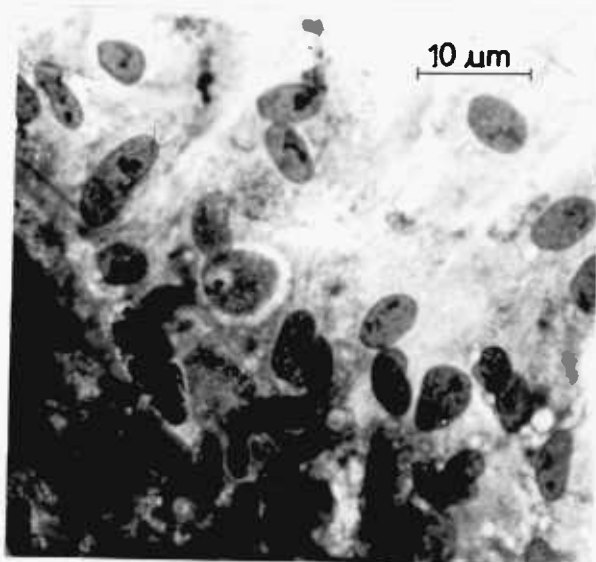
Figure 3.1.26

Immature macrogamete 7 days p.i.

Liberated second generation merozoites (average size 11.8 x 1.5µm) re-invaded cells and developed into gametocytes. The macrogamete could be identified early in its development by the persistance of a single nucleus. The nucleolus was characteristically surrounded by a clear intra-nuclear area (Fig. 3.1.26). Later development of the macrogamete was not observed.

Microgametocytes were more difficult to distinguish from schizonts in the early stages of their development. However, as they grew they could be identified by their nuclei which were compact as opposed to vesicular and became more numerous and more closely packed (Fig 3.1.27). Sometimes cytoplasmic masses resembling the blastophores of schizonts were separated from the microgametocytes. Each mass contained several nuclei (Fig. 3.1.28).

Mature microgametocytes were identified by the presence of flagellated microgametes attached to the periphery, giving a 'wispy' outline to the structure (Fig. 3.1.29). Some exceptionally large microgametocytes (70.8 x 28.3 µm) could have resulted from the fusion of two or more from adjacent cells after rupture of the parasitophorous vacuoles. No free microgametes were observed.

Although macrogametes and microgametocytes were sometimes observed in close proximity, they were never seen within the same host cell (Fig 3.1.30).

Some second generation merozoites appeared to have developed into a third generation of schizonts after invading new cells, and not into gametocytes. These schizonts were small and contained only 5-10 merozoites, intermediate between those of the first and second generations (Fig. 3.1.31a and b) with respect to size.

20 um
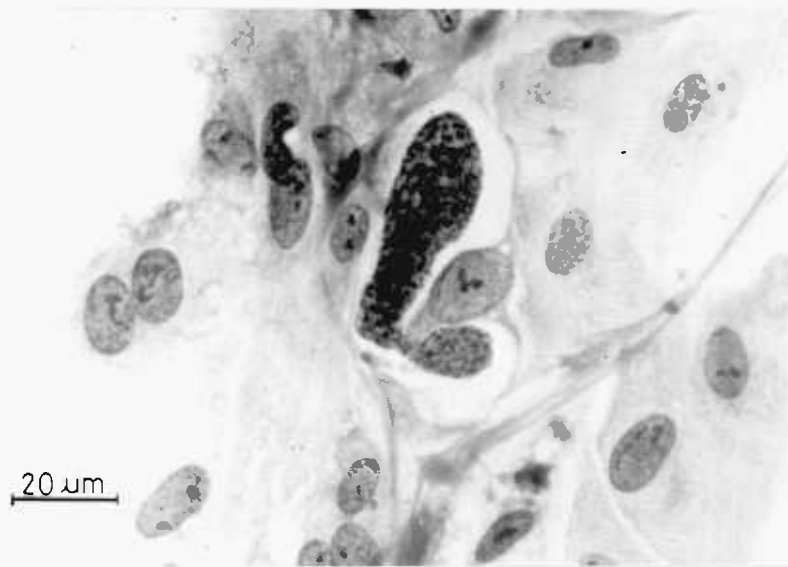
Figure 3.1.27

Immature microgametocyte 6 days p.i.

10 um
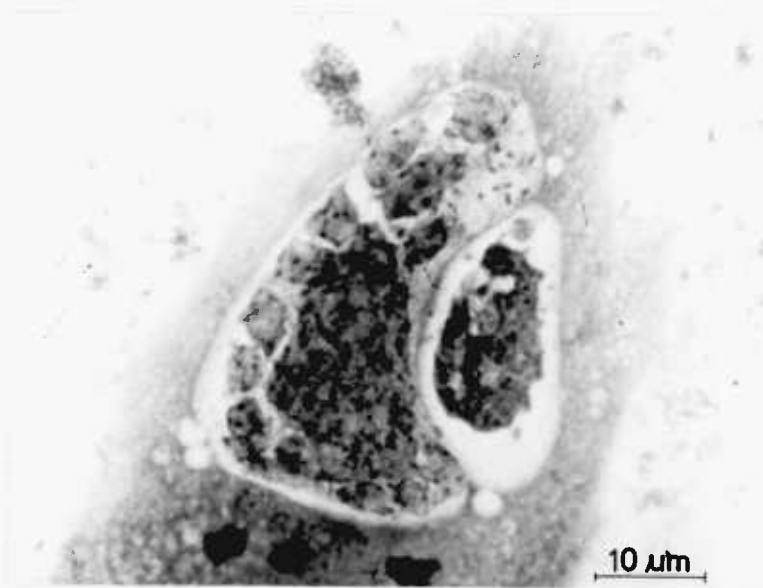
Figure 3.1.28

Immature microgametocyte; the body of the microgametocyte has divided
into several smaller parts
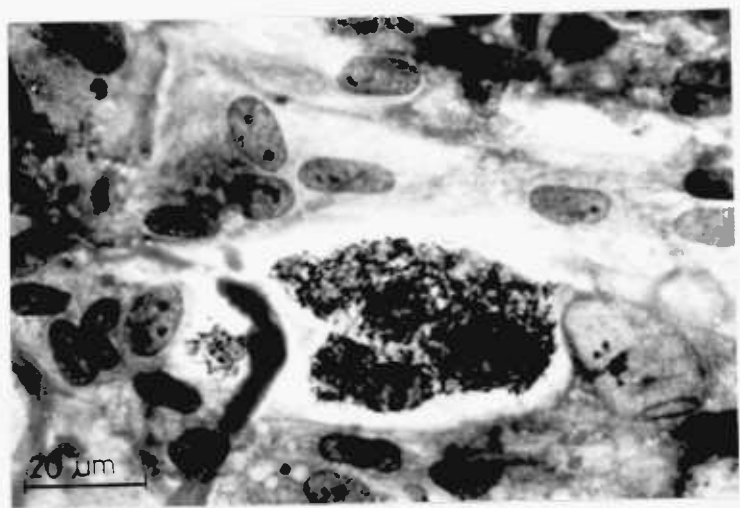
Figure 3.1.29

Mature microgametocyte
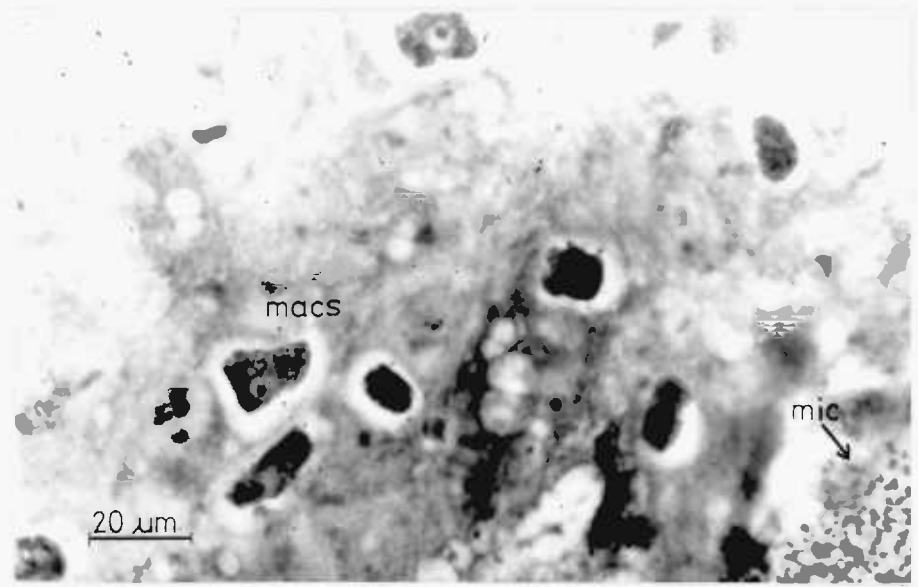
Figure 3.1.30

The association of macrogametes and microgametocytes growing in CK cells

Figure 3.1.31 Third generation schizonts of E. tenella growing in CK
cells 7 days p.i.
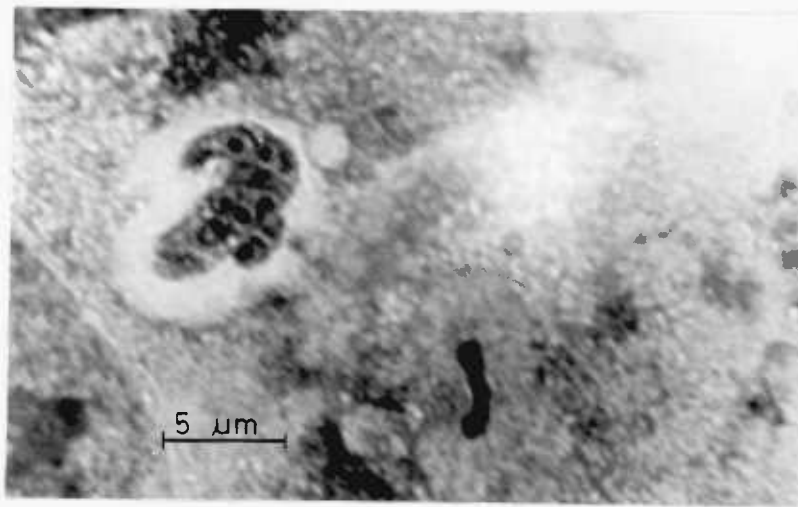
a) Immature schizont
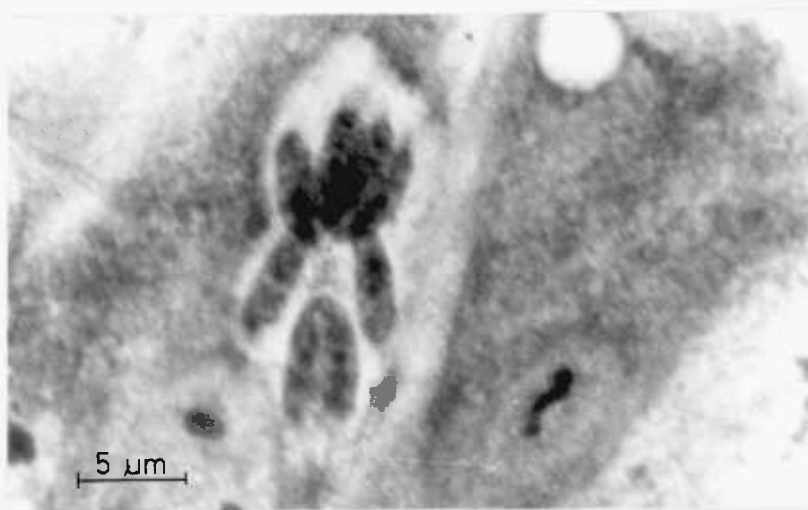
b) Mature schizont

3.1.4   Development of E. tenella in irradiated CK cells

CK cells irradiated from a Cobalt 60 source and subsequently infected with E. tenella sporozoites (2×10^5/ml) showed no initial difference in their ability to support the development of the parasite (Fig. 3.1.32). However, by day 4 p.i. the irradiated cells were less able to support the development of the parasite. Both cultures had a healthy appearance. The difference at 4 and 5 days was statistically reliable on a two way analysis of variance (F test, $p < 0.05$).

In order to ascertain if the failure of the parasites to develop was due to the inability of the irradiated cells to take up ^{3}H-thymidine (and therefore undergo DNA synthesis), both cultures were pulsed for 1 hour on day 5 p.i. with a solution of ^{3}H-thymidine at 2μCi/ml. The coverslips were processed for autoradiography and the percentage of labelled nuclei was recorded (Table 3.1.6).

Table 3.1.6   Labelled nuclei in irradiated and non-irradiated CK cells,
5 days p.i., following a 1 hour pulse with ^{3}H-thymidine

Irradiation	% labelled nuclei
5200 rads.	4.5 ± 1.30
Normal cells, no irradiation	12.7 ± 1.9

It is evident from this result that some of the cells in the irradiated population were still able to take up thymidine. However, the level was much lower than in non-irradiated cells, suggesting that overall the population of irradiated cells was less able to undergo DNA synthesis.

Fig. 3.1.32. Growth of E. tenella in irradiated CK cells.

x——x   Non-irradiated cells

o— —o   Irradiated cells
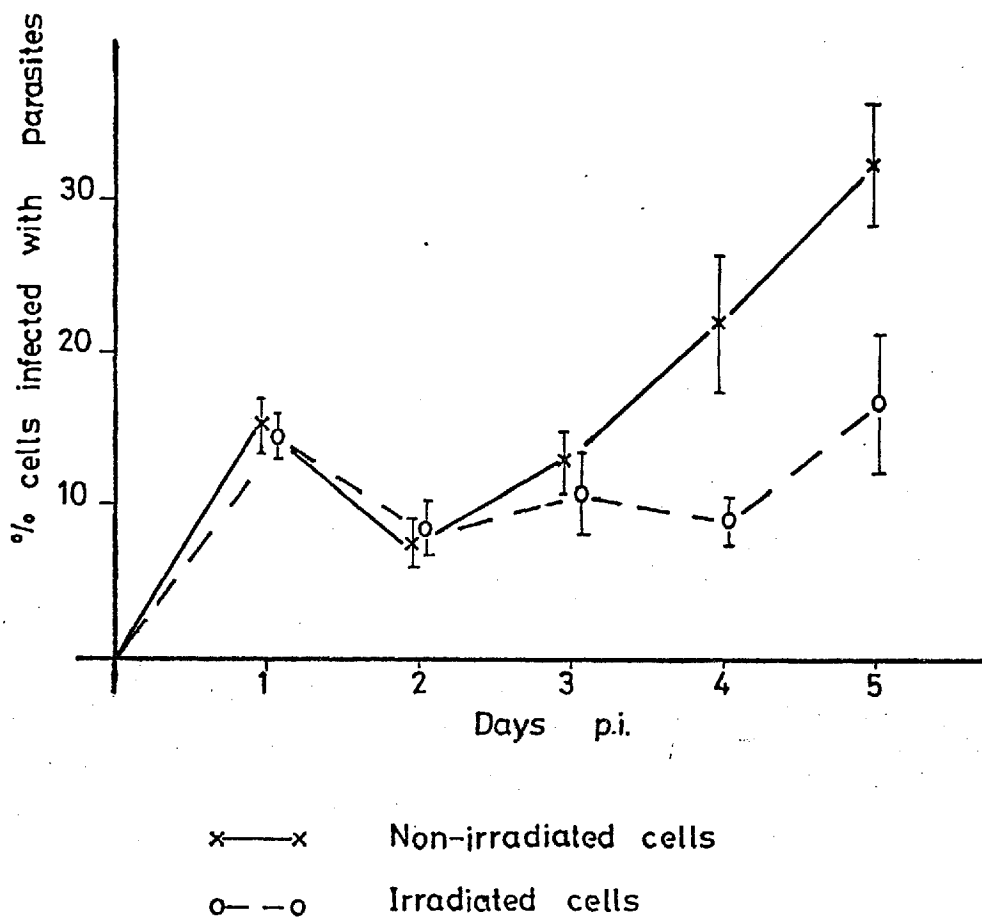
3.1.5 Development of three strains of E. tenella in CK cells
_____

Three strains were compared:-

Et(w) = Laboratory strain, sensitive to Pancoxin/

Amprolium and De$\overset{c}{q}$ox.

Dec 40 = Resistant to De$\overset{c}{q}$ox at 40 p.p.m.

Kf$_2$ = Resistant to Pancoxin at 125 p.p.m. (Pancoxin

contains 18% amprolium, 10.8% sulphaquinoxaline,

0.9% ethopabate.

The three strains of oocysts were fed to chicks, at 5,000 sporulated

oocysts/bird and the number of oocysts appearing in the faeces of each

bird was counted from days 5 to 9 p.i. On day 9 the caeca were

removed and the oocysts present there were counted and added to the

total. Development between the three strains was also compared in CK

cultures.

Kf$_2$ was responsible for the greatest oocyst output from birds.

In tissue culture Kf$_2$ also appeared better able to grow as on day 7

p.i. 13% of the intracellular stages present were gametocytes, whereas

the values for Et (w) and Dec 40 strains were 5% and 8%, respectively

(Table 3.1.7). Oocysts of Kf$_2$ strain were seen in CK cells on 8 days

p.i. (Fig. 3.1.33); this was the only occasion when oocysts were

positively identified.

Table 3.1.7 Comparison of the infectivity in vitro and in vivo

of three strains of E. tenella

Strain	Cell Culture			Chickens
	Parasitic stages at day 7p.i. (%)			
	Second generation schizonts	gametocytes	others*	Oocyst production (x10^6) day 5-9p.i.
Et(w)	78	5	17	39.3 $\pm$ 4.97
Kf$_2$	49	13	38	55.49 $\pm$ 5.41
Dec 40	69	8	23	43.61 $\pm$ 3.53

* stages difficult to identify.

The morphologies of the intracellular stages of the 3 strains and their

general development was similar to that described in Section 3.1.2vii.

3.1.6 Development of E. necatrix in CK cells from a sporozoite inoculum

CK cells were used in these experiments and no development beyond

intracellular sporozoites was observed.

3.1.7 Development of E. necatrix in cell culture from a merozoite inoculum

Intracellular schizonts from the intestinal wall (Fig. 3.1.34a) of

E. necatrix infected chickens were digested and the preparation of free

merozoites (Fig. 3.1.34b) inoculated on to both CK cells and mouse

macrophages, at a concentration of 4 x 10^5 merozoites per coverslip.

No intracellular merozoites or further development was observed, despite

many merozoites being visible over the surface of the cultures, (Fig. 3.1.35).

By day 2 p.i. both uninfected and infected cultures were deteriorating,

although they remained free of any apparent contamination.

20 um
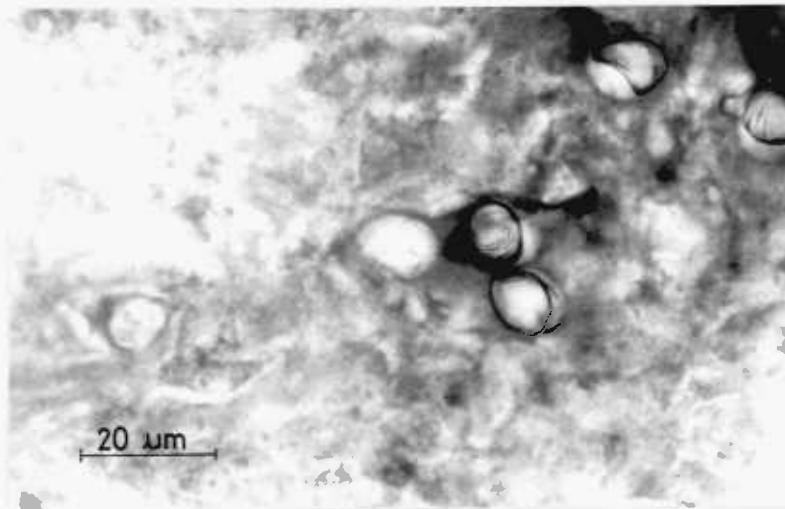
Figure 3.1.33

Oocysts of E. tenella (Kf$_2$ strain) growing in CK cells, 8 days p.i.

Figure 3.1.34 Intracellular schizont (a) and free merozoites (b)
of E. necatrix used to inoculate on to mouse
macrophage cultures (Nomarski interference microscopy)

a)

b)
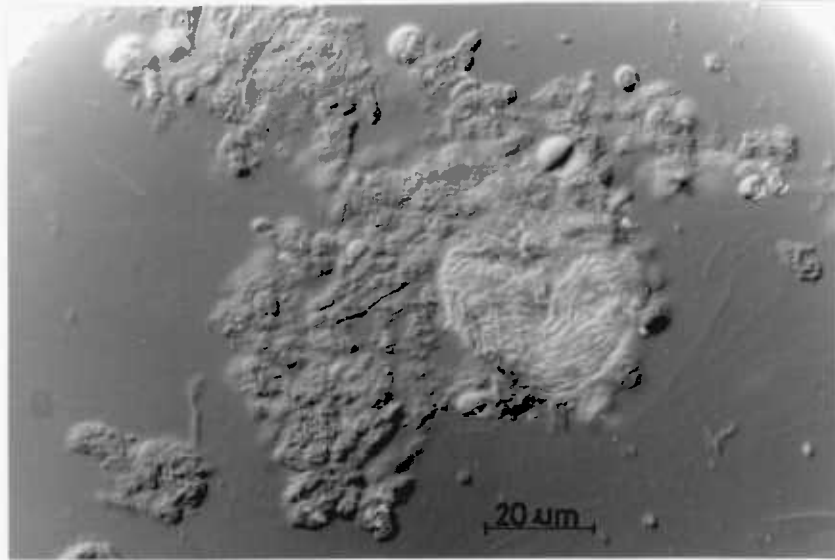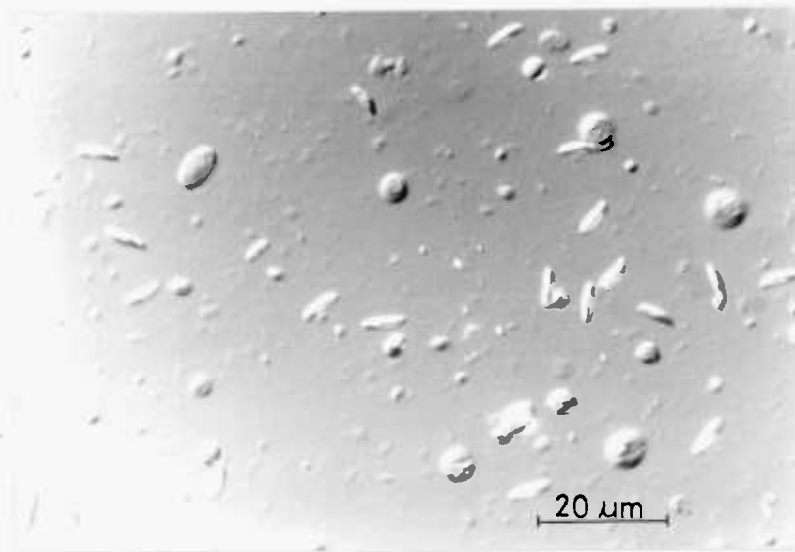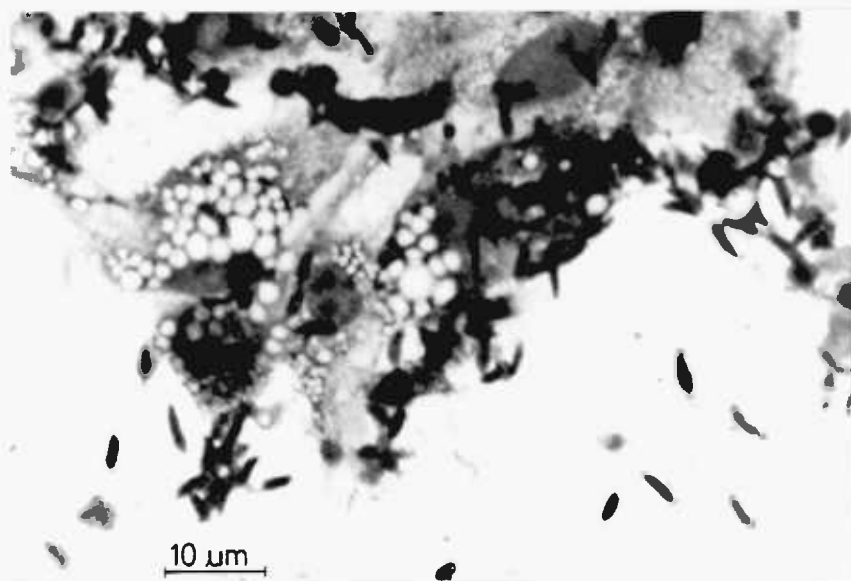
Merozoites of _E. necatrix_ 24 hours after inoculation
on to CK cultures (41°C)

3.2   Invasion of E. tenella sporozoites into CK cells

3.2.1   General Observations

When sporozoites of E. tenella were inoculated in serum-free

HBSS there was rapid invasion and by thirty minutes after inoculation

they could be detected within the cells. There was a steady increase

in the number of cells invaded up to a maximum at 10 hours (Fig. 3.2.1).

In some experiments it was necessary to store the sporozoites in buffer

at 4°C for up to 24 hours before they were used. The infectivity of

sporozoites was not lost even after 30 hours storage at 4°C (Table 3.2.1).

Table 3.2.1   The invasion of E. tenella sporozoites into CK cells

after storage for 30 hours at 4°C

% invaded cells	Incubation time at 4°C
24.77 $\pm$ 2.13	0 hours
25.67 $\pm$ 1.69	30 hours

3.2.2   The effect of serum on E. tenella sporozoite invasion into CK cells

3.2.2i   New Born Calf Serum

Sporozoites (2 x 10^5/ml) were inoculated on to the cells in HBSS

containing 1,5% and 10% new born calf serum (NBCS) which was either

normal or heat-inactivated. The number of invaded cells at 4,12 and

24 hours p.i. is recorded in Fig. 3.2.2. When the inoculation medium

was serum free, over 40% of the cells were infected at 4 hours and

there was very little increase after this time. With the addition of

normal 1% NBCS there was a slight fall in the percentage of cells

invaded at 24 hours (38.0 $\pm$ 1.50 compared with 45.53 $\pm$ 2.94 for the

control (no serum) cultures). With the higher levels of serum this

Fig.3.2.1. Invasion of CK cells by E.tenella sporozoites over 24 hours.
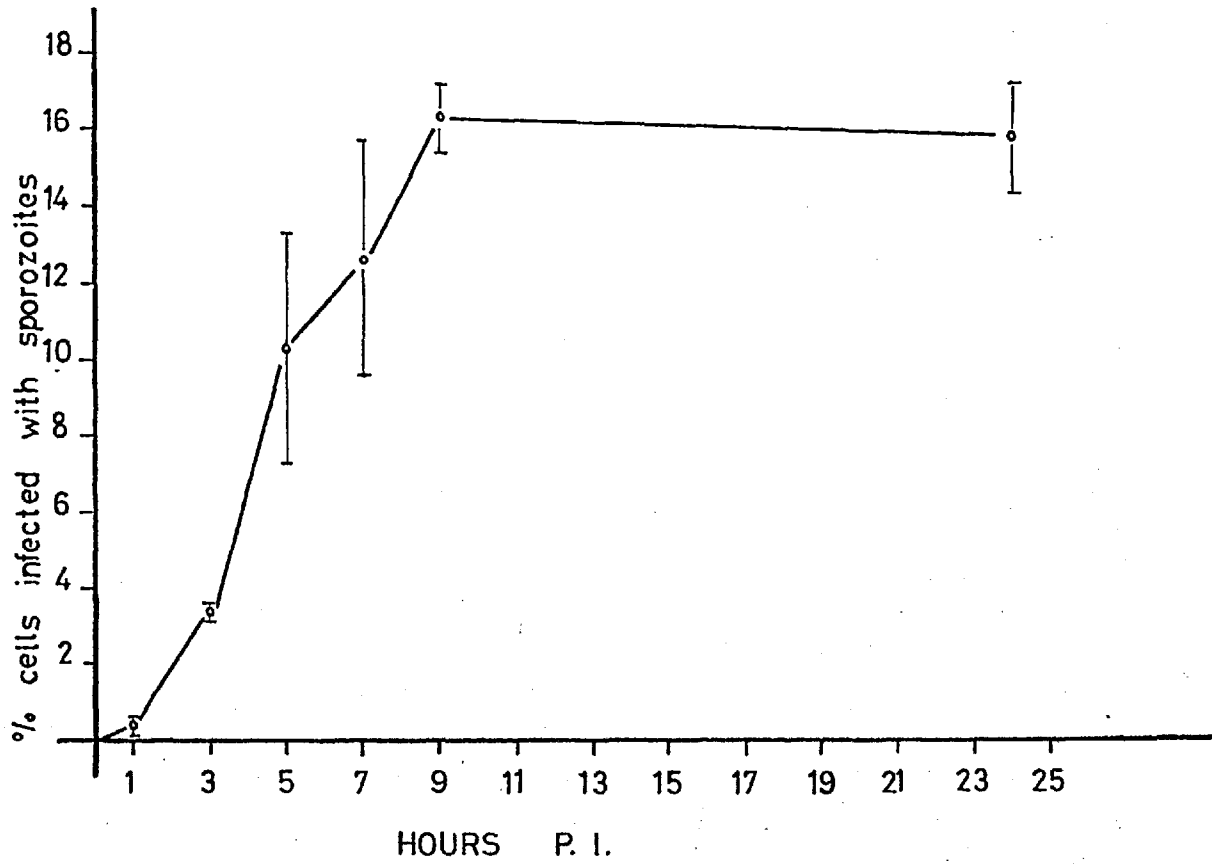
Fig. 3.2.2. Invasion of CK cells by E.tenella sporozoites in the presence of New-Born Calf Serum.

1% SERUM

5% SERUM

10% SERUM

Hours p.i.

% CELLS INFECTED WITH SPOROZOITES

x——x no serum

normal serum

heat-inactivated serum
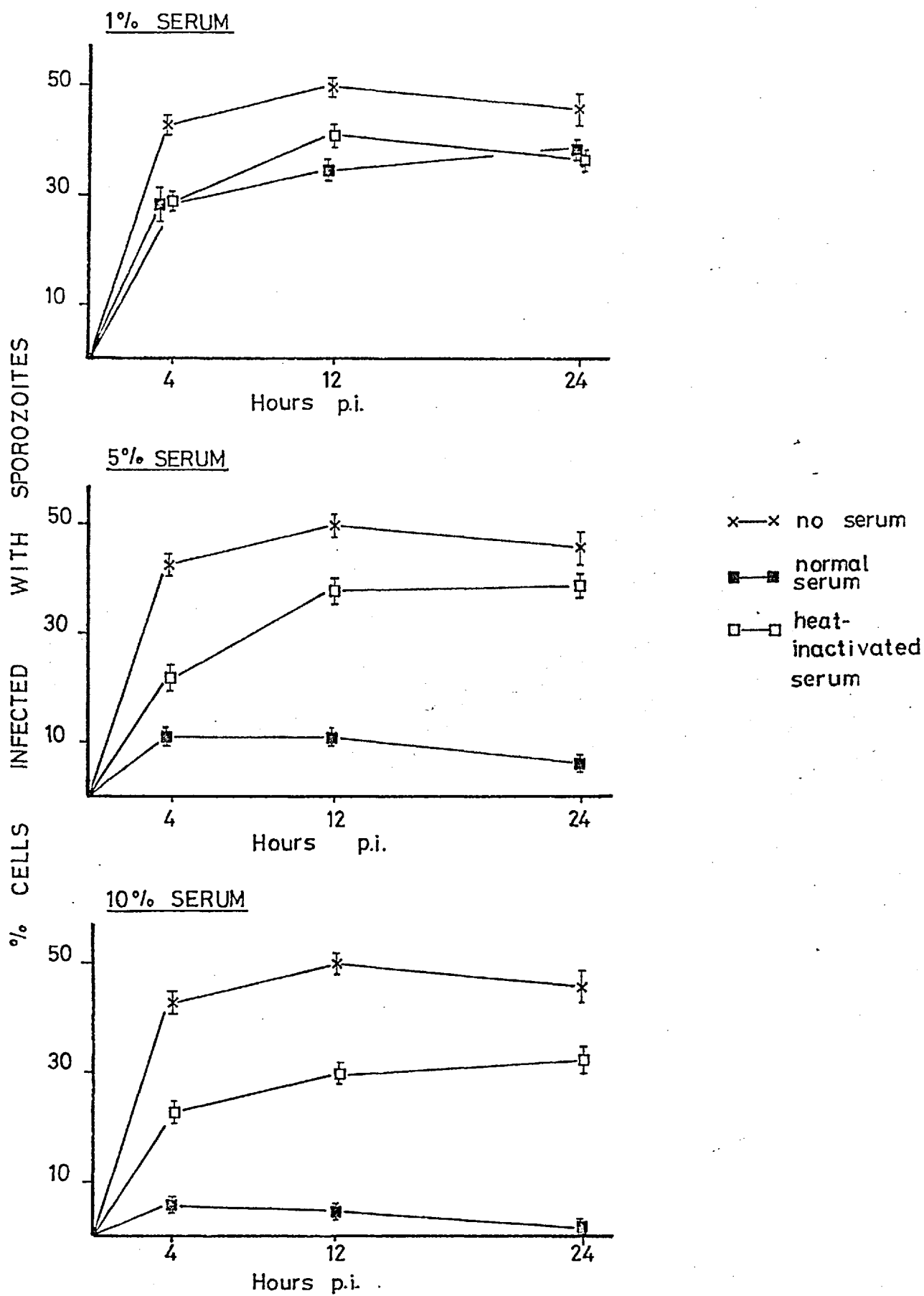
difference became increasingly obvious, with the percentage of cells
invaded at 24 hours being 6.07 $\pm$ 0.74 with 5% NBCS and 1.07 $\pm$ 0.12
with 10% NBCS. These differences were statistically reliable on a two
way analysis of variance (F test, p <0.001).

When the NBCS was heat-inactivated the inhibitory effect observed
at the higher concentrations was partially removed. After 24 hours,
sporozoites inoculated in 5% heat-inactivated NBCS had invaded
38.4 $\pm$ 1.90% of the cells compared with 45.3 $\pm$ 2.94% infected cells in
those cultures with a serum-free inoculum. With 10% heat-inactivated
NBCS some removal of invasion inhibition also occurred: the number of
invaded cells was 25% less than those in serum-free cultures, compared
with almost 100% less which had occurred with an inoculum containing
normal NBCS. However, a two way analysis of variance established that
at all levels of heat-inactivated NBCS invasion was still significantly
lower than that observed with serum-free inoculation medium (F test,
p <0.001). With respect to time, a two way analysis of variance
indicated that it was of no significance at 0,1,5 or 10% levels of
normal NBCS (F test, p >0.05), but that invasion in the presence of
1,5 or 10% heat-inactivated NBCS was always lower at 4 hours than at
12 and 24 hours p.i. (F test, p <0.01).

NBCS was also inactivated by acid-treatment and the inhibitory
effects of this serum on invasion were compared with those of normal
and heat-inactivated at 24 hours p.i. (Fig. 3.2.3). Treatment of the
NBCS with either acid or heat resulted in a very similar pattern of
invasion inhibition, which differed from that of normal NBCS. At the
5% and 10% levels, invasion in the presence of normal NBCS was
considerably lower than that recorded for similar levels of acid-treated

Fig 3.2.3. Invasion of CK cells by E.tenella sporozoites in the presence of inactivated New-Born Calf Serum.

o . . . . o normal serum

+————+ heat-inactivated serum

□ — —□ acid-treated serum
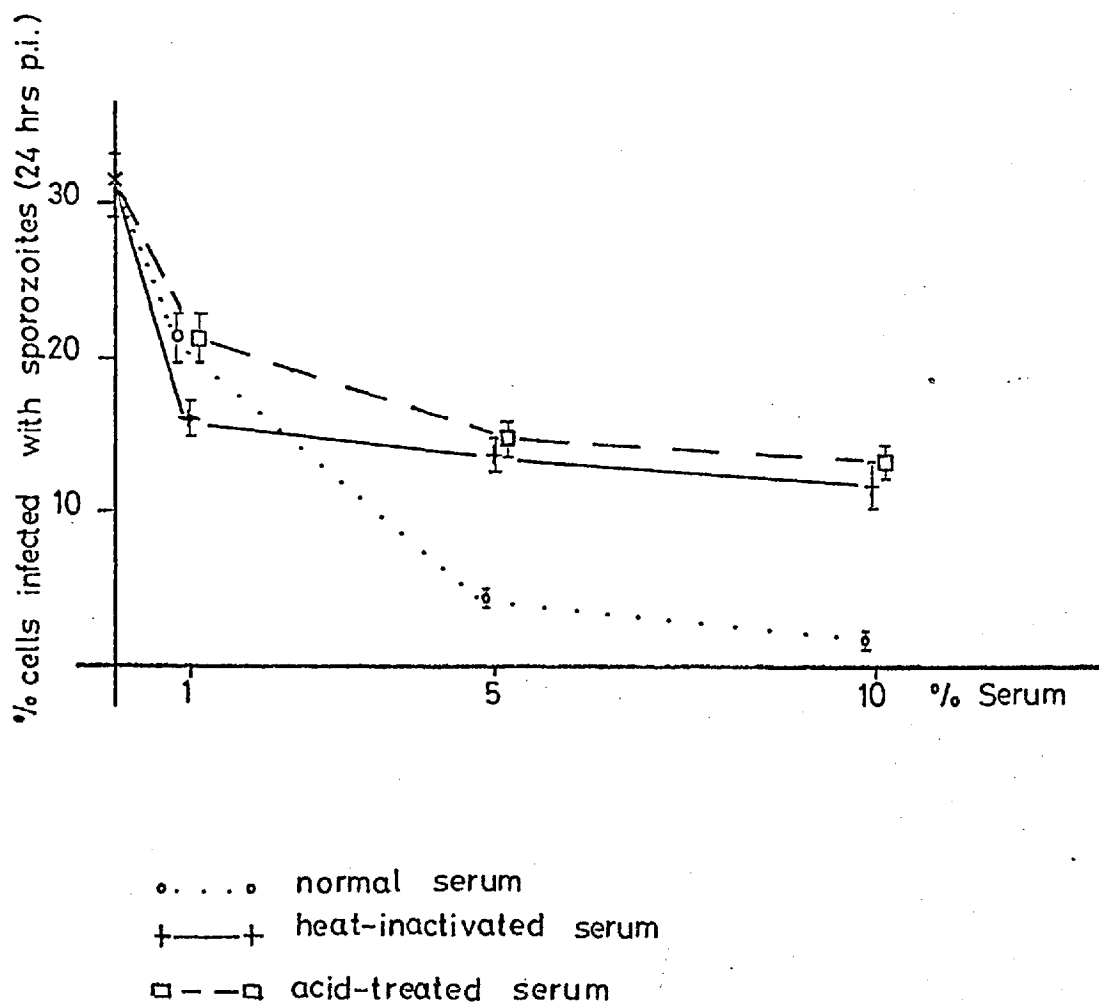
or heat-inactivated NBCS. These differences were statistically reliable on a two way analysis of variance (F test, $p < 0.001$).

3.2.2ii Donor horse serum

Donor horse serum (DHS) was tested at 1% and 10% for its inhibition of the invasion of E. tenella sporozoites (2×10^5/ml) into CK cells. Normal, heat-inactivated and acid-treated DHS were all compared and the resulting invasion at 24 hours p.i. under these various conditions is given in Fig. 3.2.4. A 45% fall in the percentage of cells invaded was observed when 1% normal DHS was added to the inoculation medium, and this inhibition was almost complete when 10% normal DHS was used. These results were comparable with the invasion inhibition obtained with normal NBCS (Fig. 3.2.2). Results from the heat-inactivated and acid-treated DHS resembled those from similarly treated NBCS (Fig. 3.2.3) in that they were less inhibitory to invasion than normal serum. However, unlike NBCS where the degree of inhibition was identical with both acid-treated and heat-inactivated serum, similarly treated DHS exhibited a difference. With 10% acid-treated serum the percentage of cells infected was 17.33 ± 0.86 and with 10% heat-inactivated it was 10.73 ± 0.83 (Student's t test, highly significant, $p < 0.001$).

3.2.2iii Chicken Serum

Fig. 3.2.5 illustrates the results from invasion studies performed with chicken serum (CS) added to the inoculum of 2×10^5 E. tenella sporozoites/ml. Normal and heat-inactivated CS at 1,5 and 10% were compared with serum-free inoculation medium. Unlike the situation with NBCS, 10% normal or heat-inactivated CS produced no lowering of the percentage of cells invaded (F test, $p > 0.05$).

Fig. 3.2.4.

Invasion of CK cells by Etenella sporozoites in the presence of Donor Horse Serum.

o·····o normal serum

+——+ heat-inactivated serum

□——□ acid-treated serum
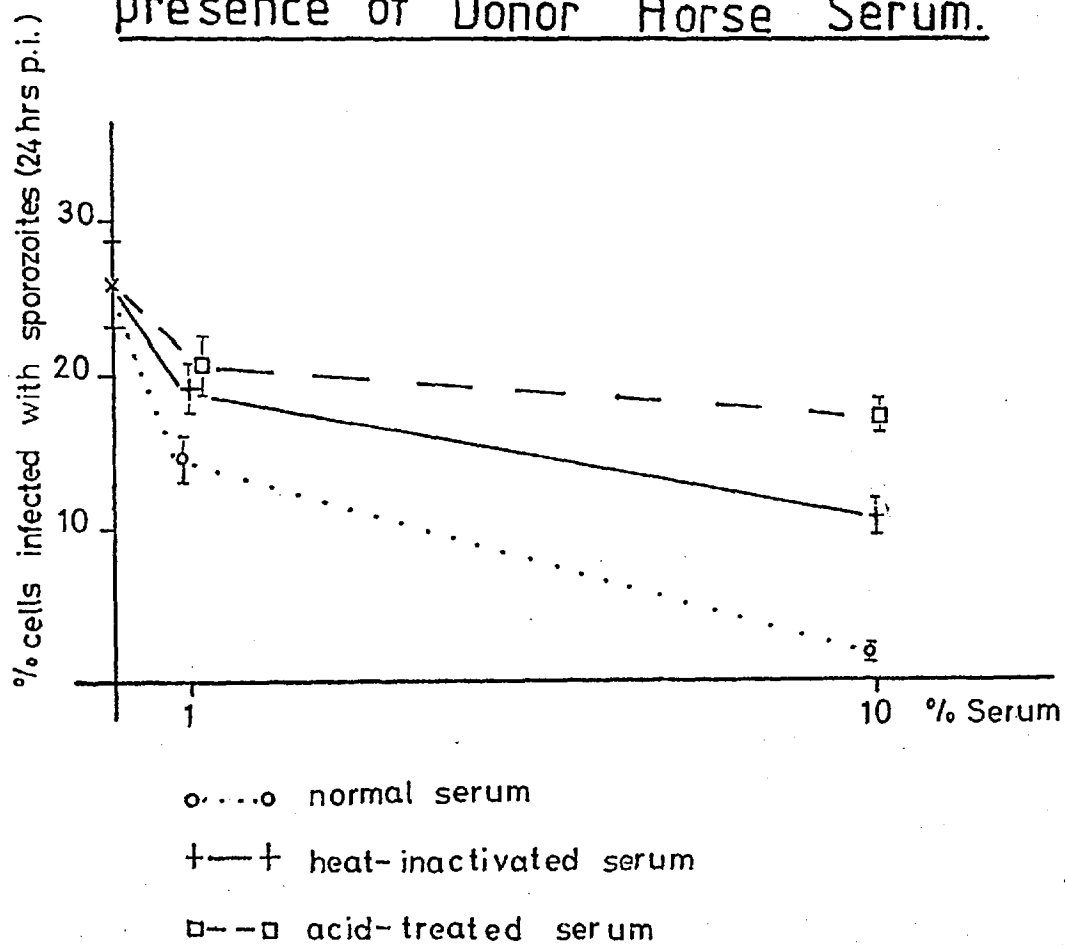
Fig. 3.2.5. Invasion of CK cells by E.tenella sporozoites in the presence of Chicken Serum.

1% SERUM

5% SERUM

10% SERUM

Hours p.i.

x——x no serum

■——■ normal serum

□——□ heat inactivated serum

% CELLS INFECTED WITH SPOROZOITES
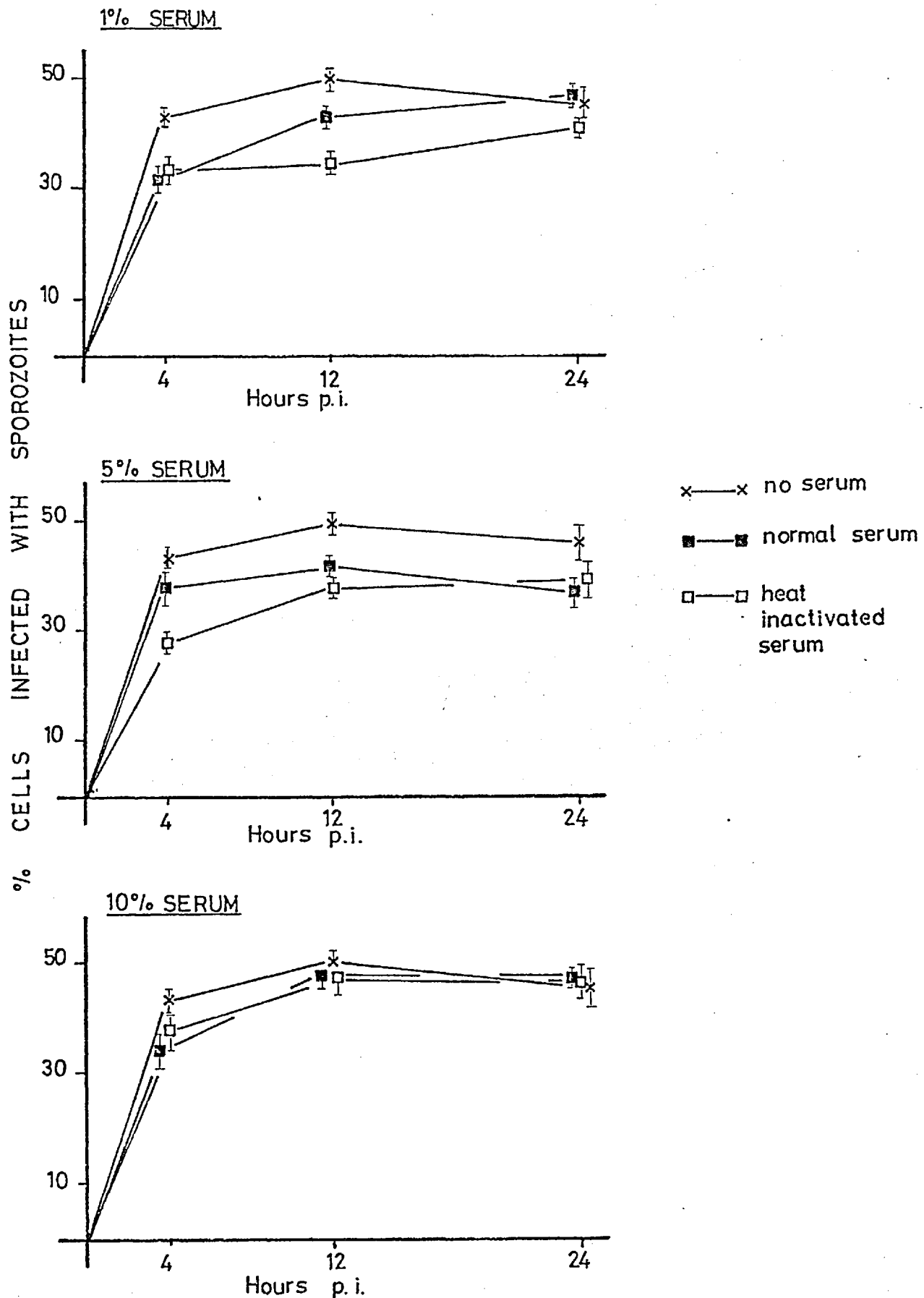
Although a slight fall in the percentage of cells invaded occurred

at 5% normal CS, the value at 1% was identical with that obtained for

serum-free inoculation medium at 24 hours p.i. With heat-inactivated

CS the percentage of cells invaded fell at both 5% and 1% (F test,

p <0.001). Invasion in CS and NBCS was identical at the 1% level,

but was greater for CS at the 5% and 10% levels.

The percentage of cells invaded for all levels of normal and heat-

inactivated CS was lower at 4 hours p.i. than at 12 and 24 hours p.i.

(F test, p <0.001). All the above results for CS were statistically

reliable according to a two way analysis of variance and p values

obtained from the F test are given in the text where appropriate.

Acid-treated CS had an identical effect on the percentage of cells

invaded to heat-inactivated and normal CS (Fig. 3.2.6).

3.2.3   The effect of protease inhibitors on invasion of E. tenella sporozoites

into CK cells

As the treatment of serum by heat or acid restricted its inhibition

on invasion, and as such treatment removes protease inhibitors (Aoki

and Kawano, 1972; Varani, Orr and Ward 1978) it was possible that protease

inhibitors could prevent sporozoite invasion into CK cells. Exogenous

protease inhibitors were therefore added to the inoculation medium,

without serum, to see if they could mimic the effects of normal NBCS on

sporozoite invasion.

3.2.3i   Soybean Trypsin Inhibitor

Soybean trypsin inhibitor (STI) was added at concentrations of

0.5, 1.0 and 2.0 mg/ml to the inoculation medium containing the

sporozoites, $(2 \times 10^5/ml)$. The invasion pattern over 24 hours was

similar in all cases (Fig. 3.2.7) but the percentage of cells invaded was

Fig. 3.2.6.

Invasion of CK cells by E. tenella sporozoites in the presence of inactivated Chicken Serum.

Legend:

o······o normal serum

+———+ heat-inactivated serum

□— —□ acid-treated serum
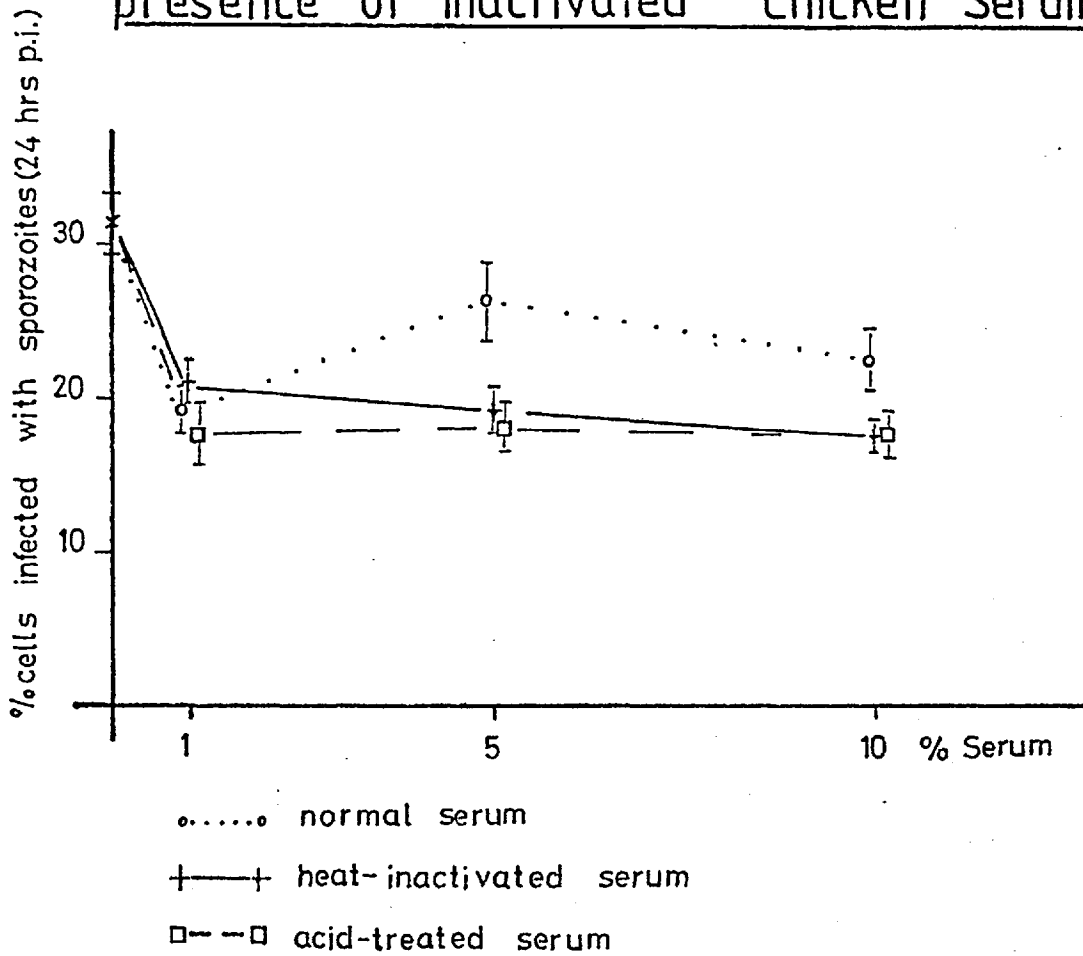
Fig. 3.2.7. Invasion of CK cells by sporozoites of E.tenella in the presence of Soybean Trypsin Inhibitor(STI)

Legend:
- ●——● no STI
- ▣——▣ 0·5 mg/ml STI
- ×···× 1·0 mg/ml STI
- +---+ 2·0 mg/ml STI

X-axis: 4, 12, 24 hours p.i.
Y-axis: % cells infected with sporozoites (10, 20, 30, 40)
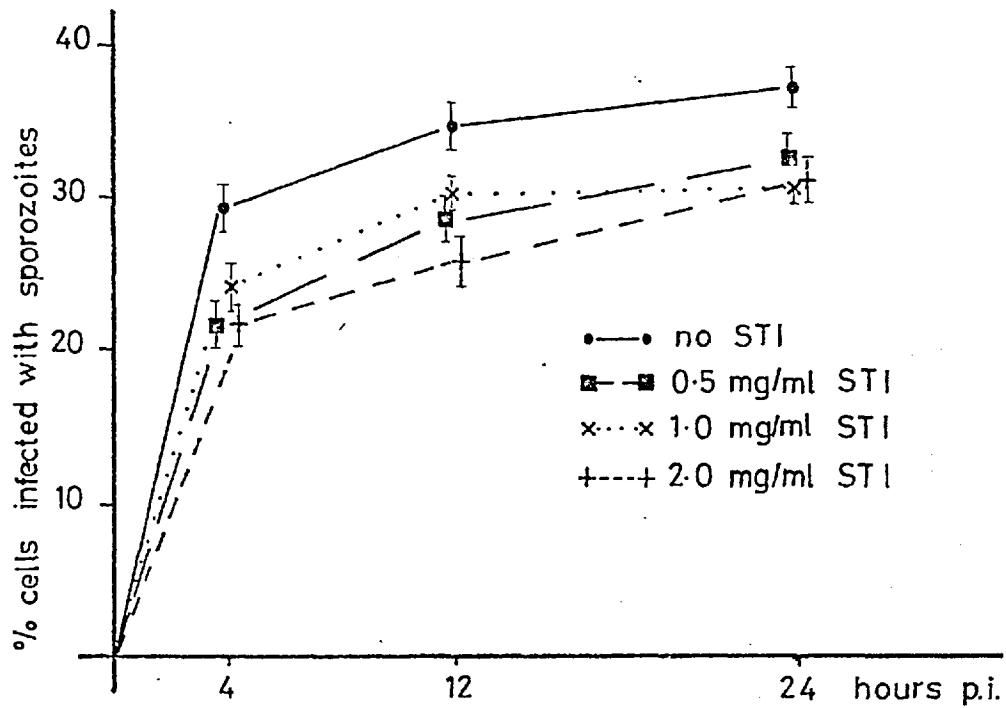
Fig 3.2.8. Invasion of CK cells by sporozoites of E.tenella in the presence of p-tosyl-l-arginine methyl ester.

Y-axis: % cells infected with sporozoites (24 hrs p.i.) (5, 10, 15, 20)
X-axis: 0.1, 1.0, 10, 100, 1000 µg/ml
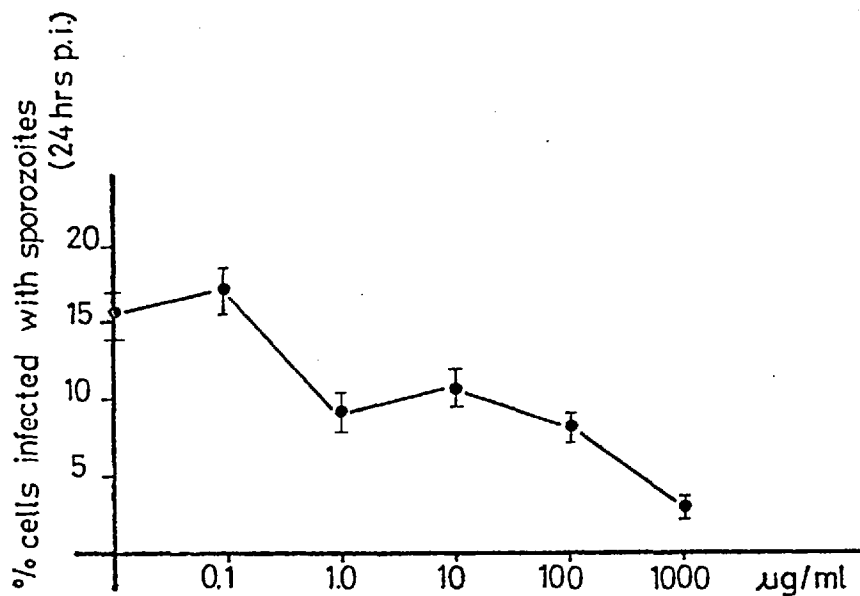
lower in all cultures where STI was present. Even at the lowest
concentration (0.5 mg/ml STI) the fall in invasion was significant
(Student's t test, p <0.025) at 24 hours p.i. and represented a
lowering of the percentage of cells infected by over 11%. Inhibition
of invasion in the presence of STI was not as complete as with some
of the NBCS levels tested (see Fig. 3.2.2).

3.2.3ii p-tosyl-arginine-methyl-ester-HCl

With increasing concentrations of p- tosyl-arginine-methyl-
ester-HCl(TAME) in the inoculation medium (containing 2×10^5/ml
E. tenella sporozoites) there was a corresponding decrease in the
percentage of cells infected (Fig. 3.2.8). At 24 hours p.i. the
percentage of cells infected in TAME-free medium was 15.6 ± 1.50.
The addition of 0.1µg/ml TAME was not inhibitory to invasion, but at
concentrations of 1µg/ml and above TAME produced a significant decrease
in the percentage of infected cells.

3.2.3iii Ovomucoid

At all of the concentrations used ovomucoid was inhibitory to the
invasion of CK cells by sporozoites when they were inoculated on to
cultures at a concentration of 2×10^5/ml (Fig. 3.2.9). At the lowest
concentration of 2µg/ml this inhibition resulted in almost a 50% fall
in the percentage of cells invaded. Increasing this concentration by
10^3 produced no significant further fall. The x^2 test indicated that
the difference observed at 2µg/ml ovomucoid was significant (p <0.005).

3.2.3iv n- tosyl-lysine-chloro-ketone-HCl

Sporozoites (2×10^5/ml) were inoculated onto CK cells in the
presence of n- tosyl-lysine-chloro-ketone-HCl (TLCK) at concentrations
of 10 and 100 µg/ml. The results obtained at 4 hours p.i. are

Fig. 3.2.9. Invasion of CK cells by _Etenella_ sporozoites in the presence of ovomucoid.
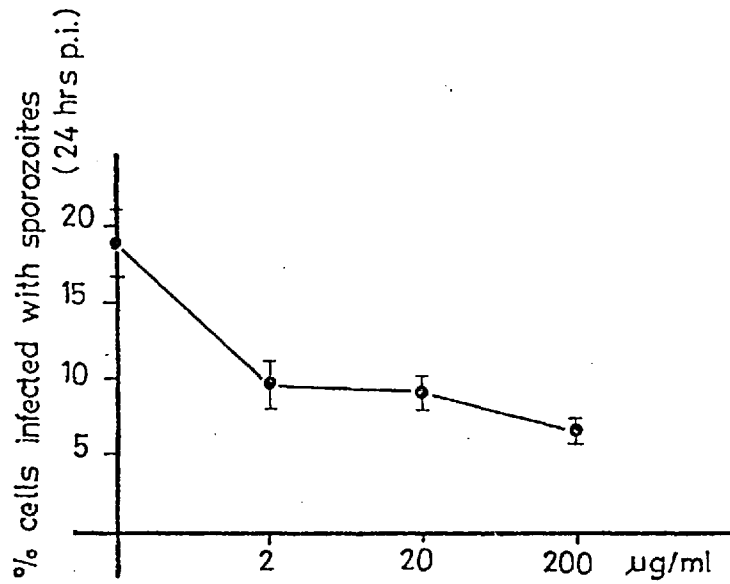
Fig. 3.2.10. Invasion of CK cells by _E.tenella_ sporozoites in the presence of N-α-tosyl-l-lysyl chloromethyl ketone.
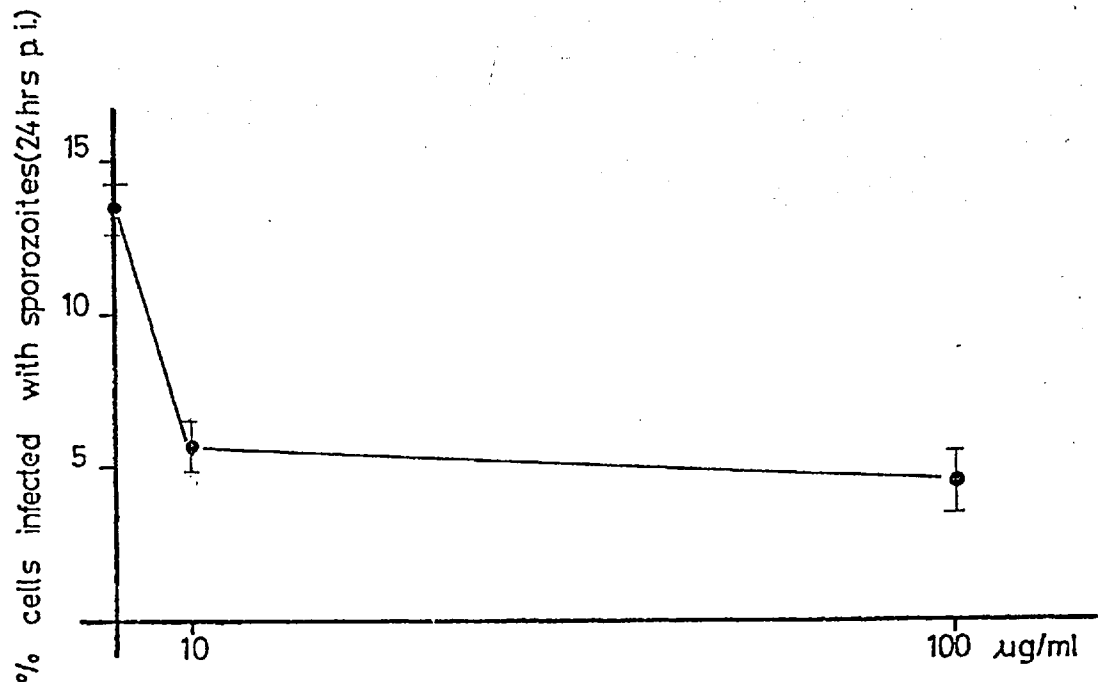
illustrated in Fig. 3.2.10. Both the concentrations were inhibitory to invasion; 10μg/ml lowered the percentage of infected cells from 13.60 $\pm$ 1.60 to 5.60 $\pm$ 0.84, a fall of over 40% (significant according to the X^2 test, p <0.005).

3.2.3v   Bovine Serum Albumin

Sporozoites of E. tenella (at 2 x 10^5/ml) were inoculated on to CK cells in the presence of bovine serum albumin (B.S.A.). The results (Fig. 3.2.11) with this chemical were rather inconclusive because although there was a reduction in the percentage of infected cells at the highest concentration (2 mg/ml) of BSA used, the response varied somewhat at the intermediate concentrations, with 1.0 mg/ml BSA producing an anomalous result. A subsequent experiment, in which this level of 1.0 mg/ml was tested again, produced a fall in the percentage of infected cells from 18.73 $\pm$ 2.29 to 6.87 $\pm$ 0.86.

3.2.4   The effect of pre-incubation of sporozoites in serum and protease inhibitors on invasion

It was possible that the apparent fall in the number of sporozoites invading cells in the presence of serum and protease inhibitors, could have been due to a decreased viability of the sporozoites in their presence, rather than to a specific effect they were having on the invasion process. To test for any such effects sporozoites (2 x 10^5/ml) were incubated in medium containing one of these compounds for 4 hours at 41°C before being washed free of the compound and added to the cells in fresh, unsupplemented medium.

The results from the pre-incubation of sporozoites in 1% and 10% normal and heat-inactivated NBCS are given in Fig. 3.2.12a and b. Values above the base line indicate a fall in the percentage of infected

Fig 3.2.11. Invasion of CK cells by E.tenella sporozoites in the presence of Bovine Serum Albumin(B.S.A.)
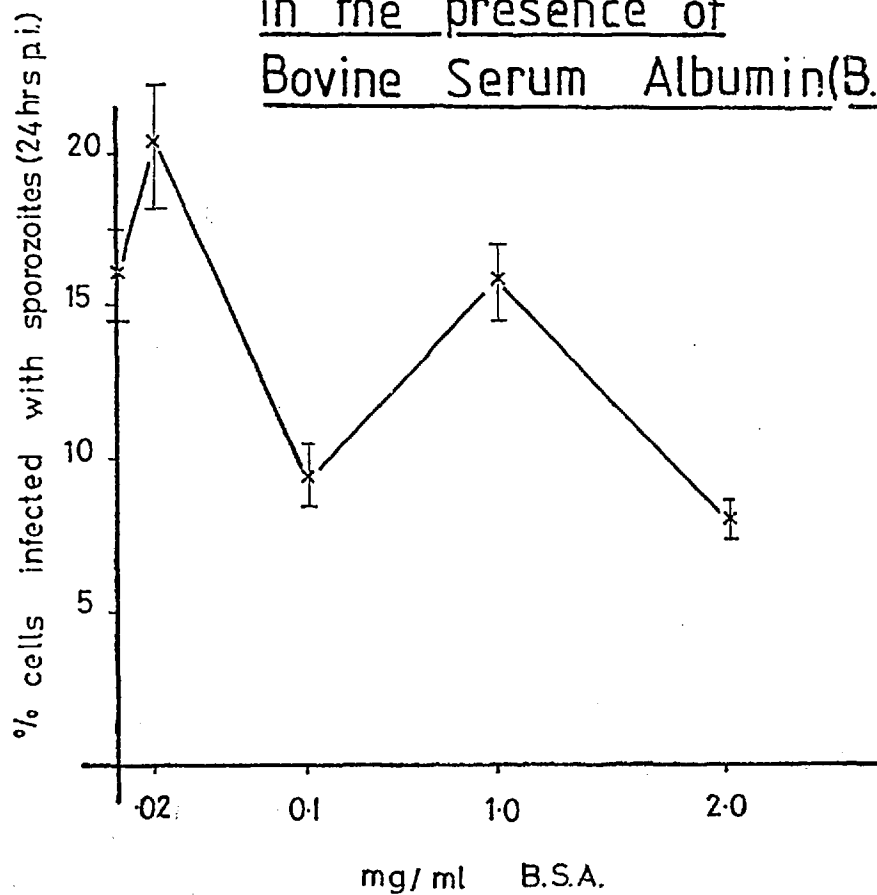
Fig. 3.2.12. Effect of pre-incubation with NBCS on the invasion of CK cells by E.tenella sporozoites.

a. No incubation.

b. Incubation — 4hours/4°C
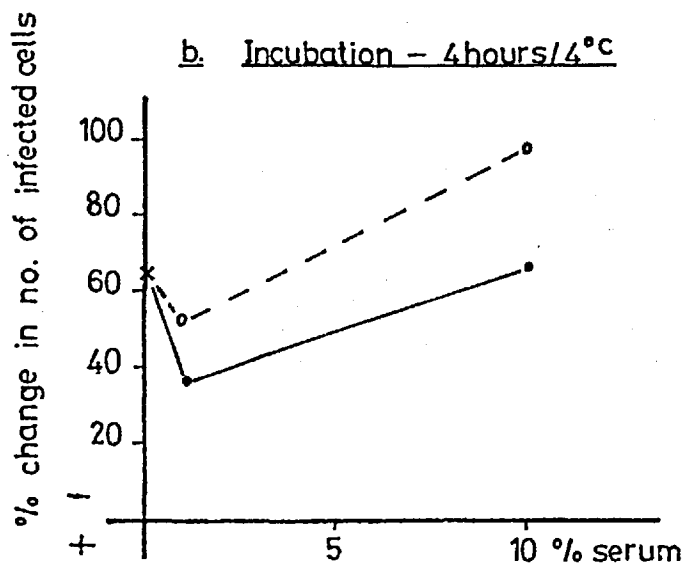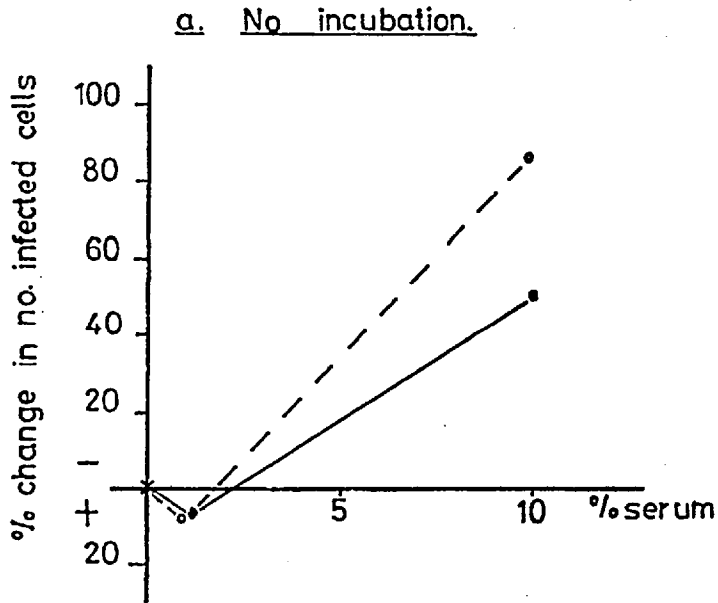
o--o normal NBCS

•—• heat-infected NBCS

cells, and those below indicate an increase in the percentage of infected cells when compared with the value recorded for invasion by sporozoites in unsupplemented medium, without an incubation.

Without incubation, sporozoites were slightly more invasive (+ 4%) in the presence of 1% normal and heat-inactivated NBCS, than in unsupplemented medium (Fig. 3.2.12a). At 10% levels of both normal and heat-inactivated NBCS, there was considerable inhibition to invasion (-87% and -51%. respectively).

Pre-incubation of the sporozoites (Fig. 3.2.12b) in the presence of 10% normal and heat-inactivated NBCS for 4 hours, lowered the invasion still further. However, as incubation in HBSS alone produced a -64% change in the percentage of infected cells, it seemed possible that the high temperature was also largely responsible for the reduction in the percentage of invaded cells after pre-incubation in the serum; the effects of serum being minor.

Results of pre-incubation of sporozoites with some of the protease inhibitors are shown in Fig. 3.2.13a and b. All three compounds TLCK, TAME and ovomucoid produced a fall in the percentage of infected cells (Fig. 3.2.13a). When incubated for 4 hours at 41°C in the highest of the concentrations the percentage of infected cells fell, (Fig. 3.2.13b). However, in the case of ovomucoid and TAME there was very little difference between the values for these compounds and the reduction seen when sporozoites were incubated in HBSS alone. TLCK produced a lowering of percentage of infected cells in excess of this.

3.2.5 The effect of hyaluronidase on invasion of sporozoites into CK cells

Sporozoites (2 x 10^5/ml) were added to CK cell cultures in medium

Fig 3.2.13. Effect of pre-incubation with protease inhibitors on the invasion of CK cells by E.tenella sporozoites.

a. No incubation.

b. Incubation-4hours/ 4°C

o····o ovomucoid

o--o TAME

o—o TLCK
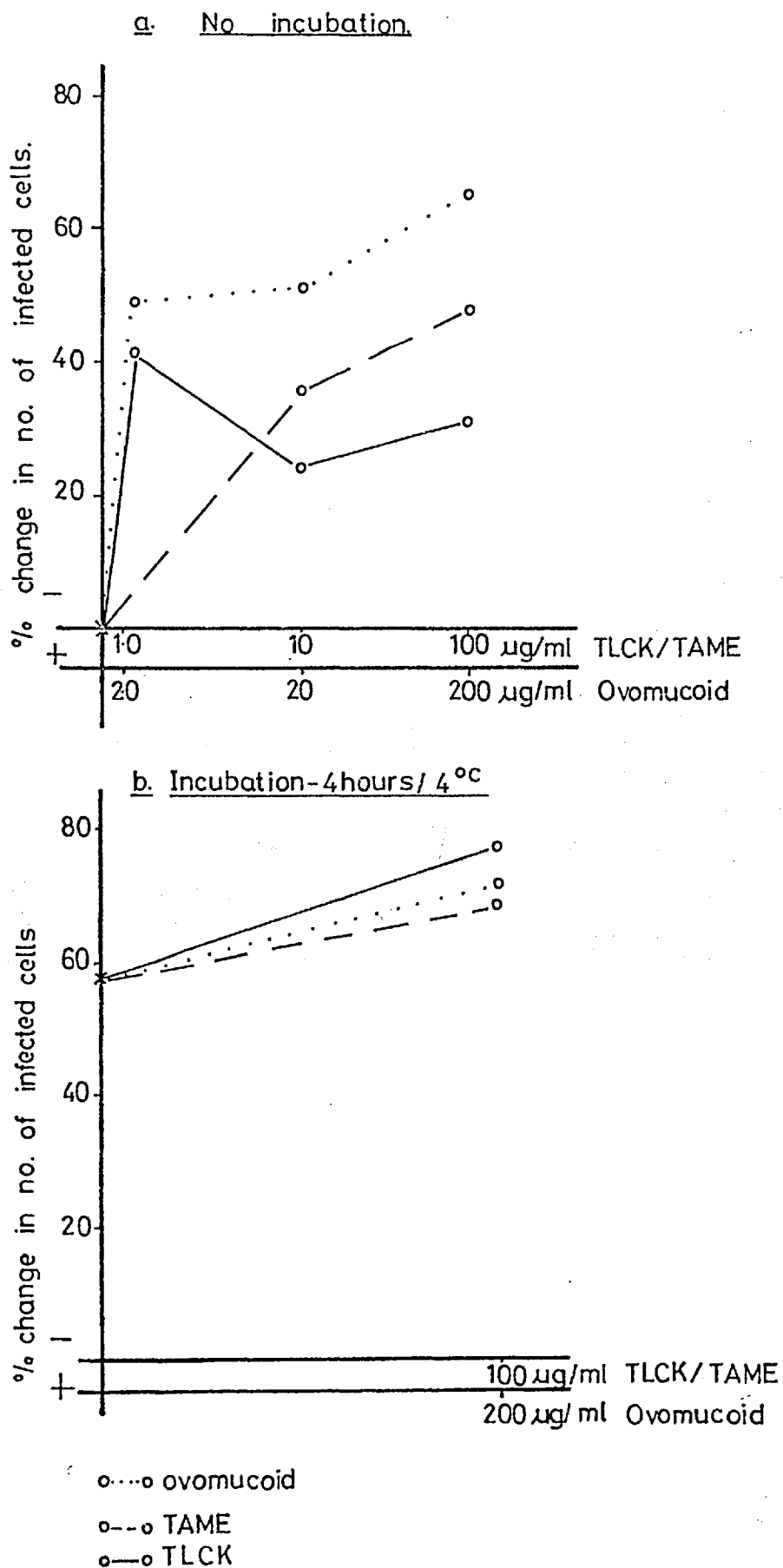
containing various levels of hyaluronidase. Concentrations above

1mg/ml lowered the percentage of infected cells (Fig. 3.2.14).

Pre-incubation with hyaluronidase for 4 hours at 41°C produced a fall

in the percentage of infected cultures in excess of that observed

for sporozoites incubated in HBSS alone (Fig. 3.2.15a and b). The

percentage change in the number of infected cells was calculated as

described in 3.2.4.

3.2.6 Invasion into synchronised CK cells

3.2.6i Assessment of synchrony

When cells are placed in low serum, their growth is arrested and

they become a synchronous G_o population. By placing the cells in a

fresh supply of 10% serum the cell cycle is reinitiated and the cells

leave G_o and pass into S phase when they take up ^{3}H-thymidine

(Mitchison, 1973).

CK cells were placed in 0.4% NBCS and released 60 hours later by

the addition of 10% NBCS. To establish that these cells were in

synchrony they were pulsed at intervals following the release from

serum starvation with HBSS containing 2μCi/ml ^{3}H-thymidine. The

radioactive solution was left on the cells for 1 hour and then they

were processed for autoradiography as described in 2.7.2i and ii.

These pulses were given at intervals over a 27 hour period following

the release from block and the results are given in Fig. 3.2.16. A

population of asynchronous cells acted as a control. They had been

given a frequent supply of fresh HBSS with 10% NBCS and were therefore

an actively growing population.

Following the addition of 10% NBCS the serum starved group showed

an increase in incorporation of ^{3}H-thymidine from 48.58 c.p.m. $\pm$ 1.30

Fig.3.2.14. Invasion of CK cells by E.tenella sporozoites in the presence of hyaluronidase.
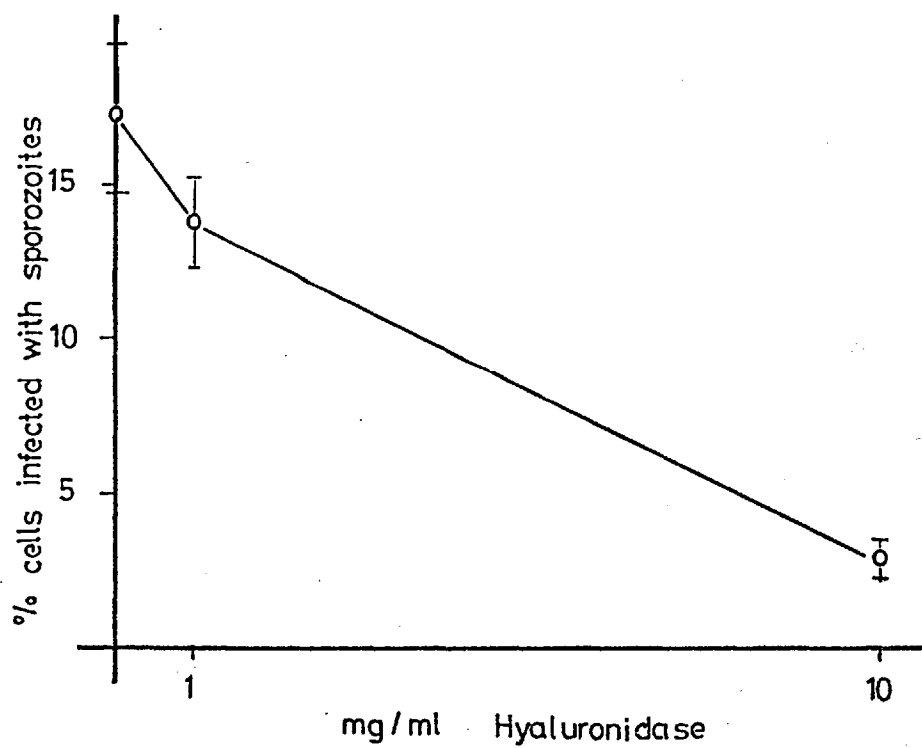
Fig 3.2.15. Effect of pre-incubation with hyaluronidase on the invasion of CK cells by E.tenella sporozoites.

a. No incubation.

b. Incubation— 4hours/4°C.
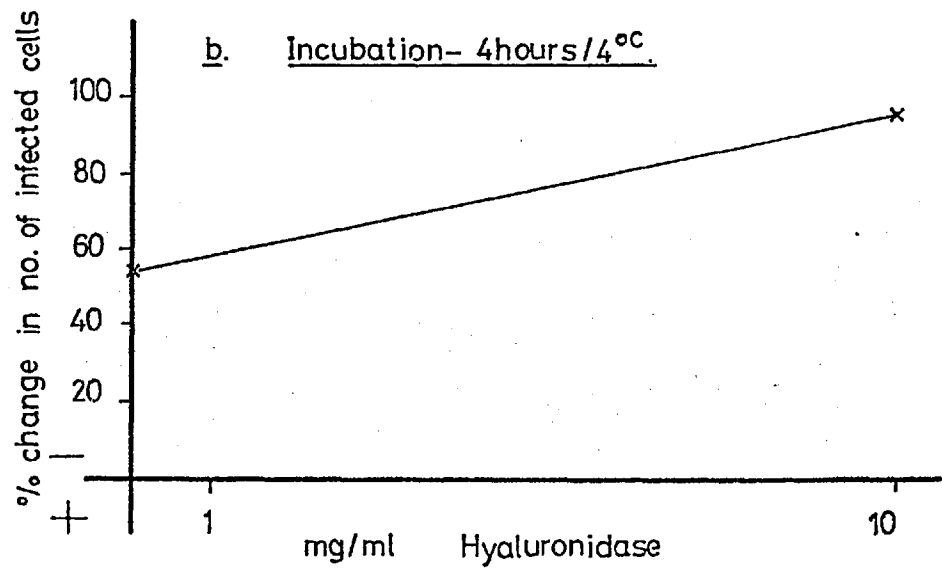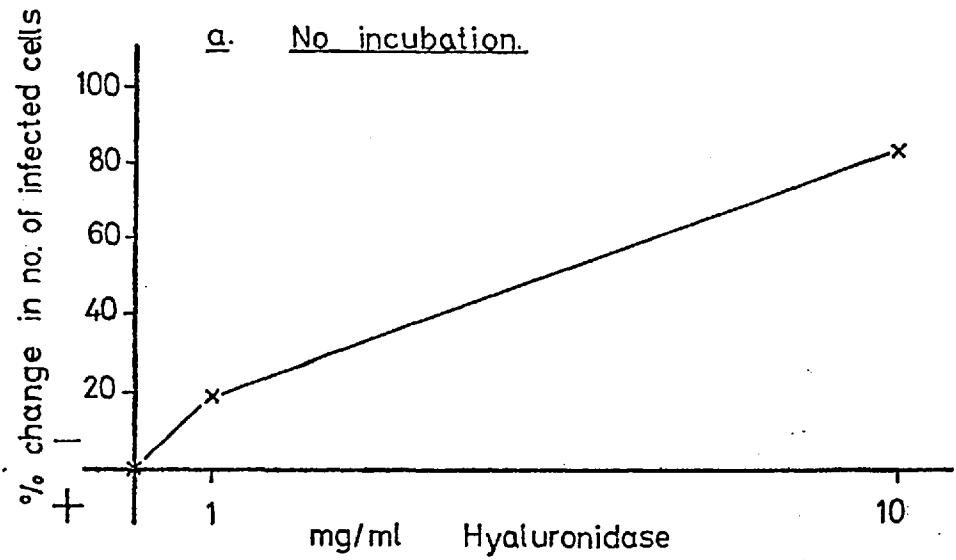
Fig. 3.2.16.

^{3}H-Thymidine uptake by synchronised CK cells in response to release from serum starvation.

Hours after release from serum starvation
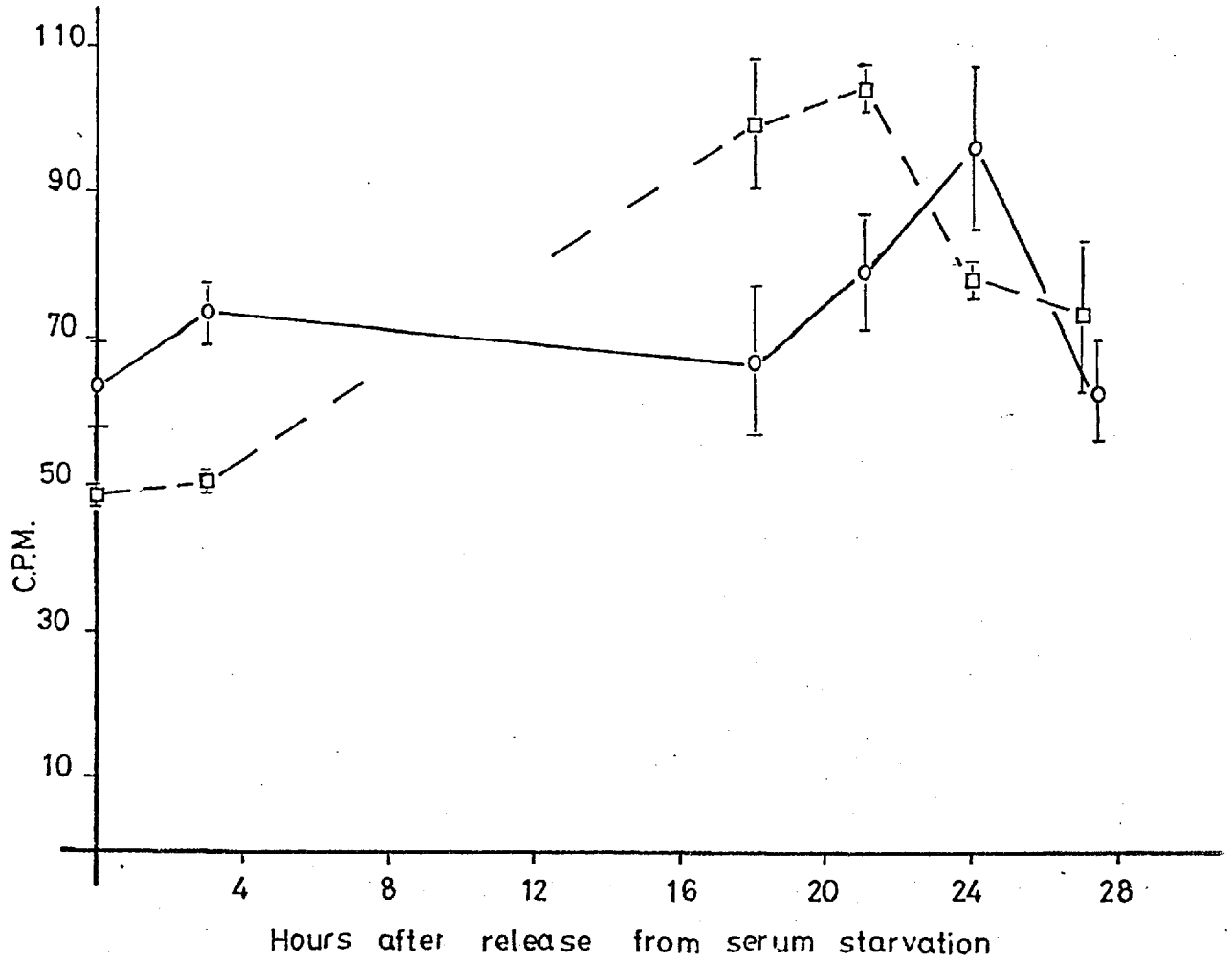
o———o   asynchronous cells

□——□   synchronous cells

as they were stimulated to leave G_o. On entering the S phase

(when thymidine is incorporated, Cleaver, 1967) the incorporation

reached a peak of 105 c.p.m. $\pm$ 3.0, representing an increase of

115%, at 21 hours after the cells had been placed in 10% serum.

With the asynchronous population there was a higher initial

incorporation level of 63.83 c.p.m. $\pm$ 5.4, and only a 51% increase

of uptake of ^{3}H-thymidine to 96.43 c.p.m. $\pm$ 11.46 which reached a

peak slightly later than the experimentals at 24 hours. The serum-

starved group were thus in synchrony and over the first 20 hours

following release contained a greater number of cells entering the

S phase than did the controls.

3.2.6ii Invasion of synchronised CK cells

Sporozoites (2 x 10^5/ml) were added simultaneously with the

release of the serum-starved cells from block. 10% serum was

necessary to initiate the stimulation of serum-starved cells. As

10% normal NBCS was inhibitory to invasion of the sporozoites

(Fig. 3.2.2.), heat-inactivated NBCS was used in preference to this

in the inoculation and stimulation media. Similar inocula were

added to normal, asynchronous cultures. Cultures were fixed at

intervals up to 4 hours post-release, and the percentage of infected

cells for synchronous and asynchronous cultures was recorded

(Fig. 3.2.17a). The difference between the two cultures was not

statistically significant (F test, p > 0.05), although there was a

tendency for the synchronised cell population to be more heavily

infected.

At the time of release from the block, a group from both the

synchronised and asynchronised cultures was not infected with

sporozoites immediately, but was left for 9 hours in medium

Fig. 3.2.17. Invasion of synchronised CK cells by E. tenella sporozoites.

a. Sporozoites added as cells released from serum-starvation.

b. Sporozoites added 9 hours after cells were released from serum starvation.

•— —• asynchronous cells   •——• synchronous cells
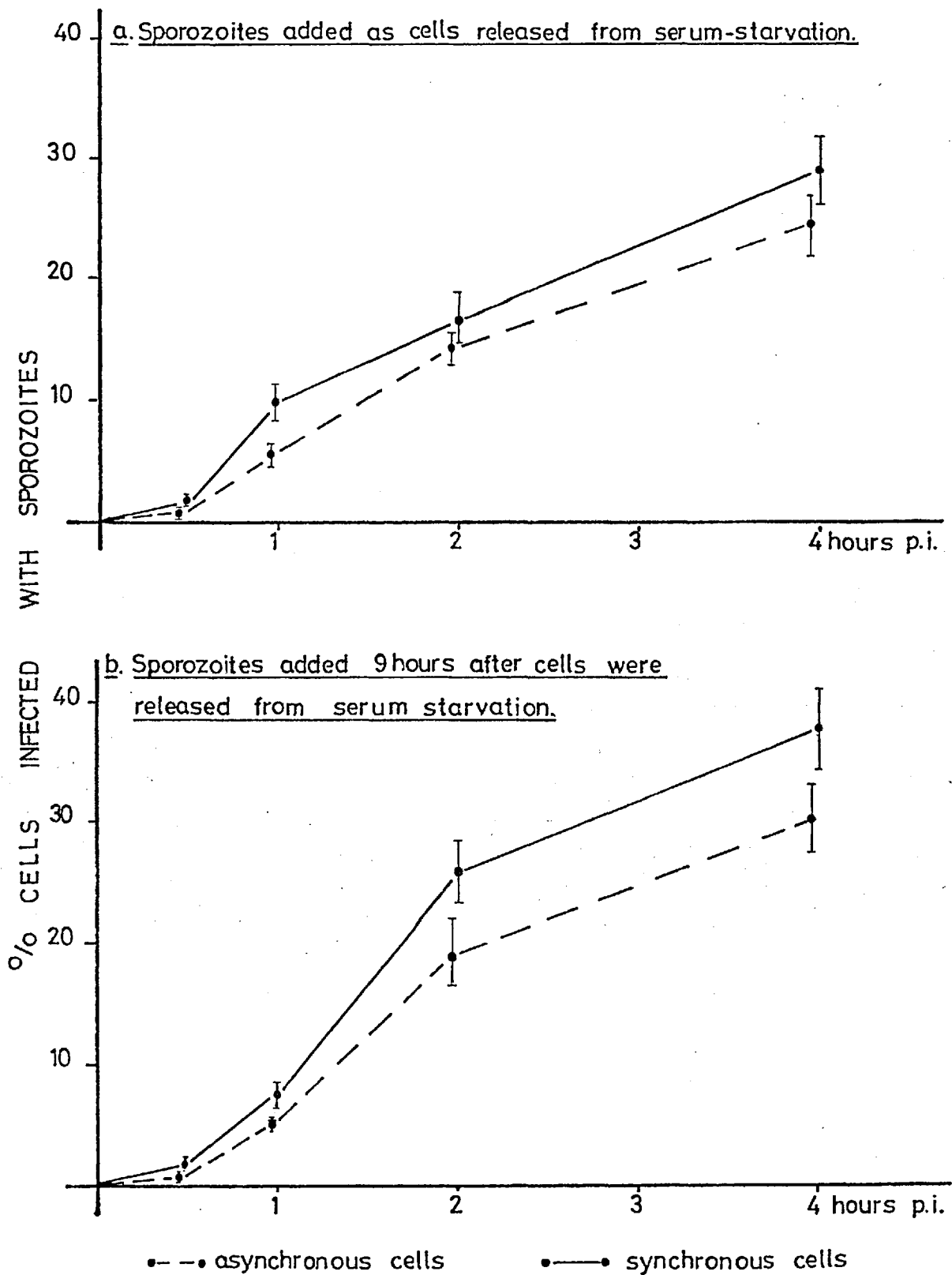
containing 10% heat-inactivated serum. After this interval had

elapsed sporozoites were added. The same suspension of sporozoites

was used, previous results (Table 3.2.1) having indicated that

storage at 4°C for up to 30 hours had no detrimental effect on

sporozoite invasion. A small difference was now apparent in the

percentage of cells invaded: there was greater invasion in the

synchronised population (Fig. 3.2.17b). This data is reliable

according to a two way analysis of variance (F test, $p < 0.05$). The

difference between the two populations did not increase over the 4

hour period shown in these results. Cells from the synchronised

population would be mainly those entering or about to enter S phase

(see Fig. 3.2.16).

3.2.7 Invasion of irradiated cells by E. tenella sporozoites

Cells which had been irradiated with 5,200 rads were inoculated

with E. tenella sporozoites, $(2 \times 10^5/\text{ml})$. The percentage of infected

cells at 24 hours p.i. was compared with that of non-irradiated cells

(Table 3.2.2). There was little difference between the ability to

invade irradiated and non-irradiated CK cells.

Table 3.2.2 Invasion by E. tenella sporozoites into irradiated

CK cells

Irradiation	Percentage of Infected Cells
5200 rads	24.87 ± 2.13
No irradiation	20.13 ± 1.30

3.3 Host Cell Changes

3.3.1 Morphological Changes

Within one day of inoculation of the parasites on to a mono-layer of CK cells the appearance of these cells by light microscopy began to alter. They appeared to be less well adhered to the coverslips, as many floated off into the overlying medium and gaps appeared in the cell layer. Fig. 3.3.1 shows the greater confluency of a non-infected culture (b) than of an infected culture (a).

These morphological changes were subsequently studied with the scanning electron microscope (SEM). In the non-parasitised cultures, the cells were growing as a confluent monolayer. All the cells showed cell to cell contact typical of this type of growth, which resulted in a pavement effect (Fig. 3.3.2). In the parasitised cultures on day 1 p.i. the cells were beginning to move apart from one another, creating gaps in the monolayer. The cells appeared to be in less close contact with the substrate and their edges were rolled back (Fig. 3.3.3).

When these groups were compared after 3 days growth of the parasite the differences were even more noticeable. Fig. 3.3.4 is a micrograph of the control group which showed very little change from the 1 day condition. In parasitised cultures (Fig. 3.3.5) the cells had moved far apart and remained in contact with one another in many places only by long cytoplasmic bridges. In several cases even these had broken down, and individual cells remained attached to the substrate by long retraction fibres. The distal ends of the retraction fibres represented the former adhesion sites of the epithelial cells to the glass. In Fig. 3.3.5c the top right hand cell

Figure 3.3.1 The appearance of CK cells, 1 day p.i.
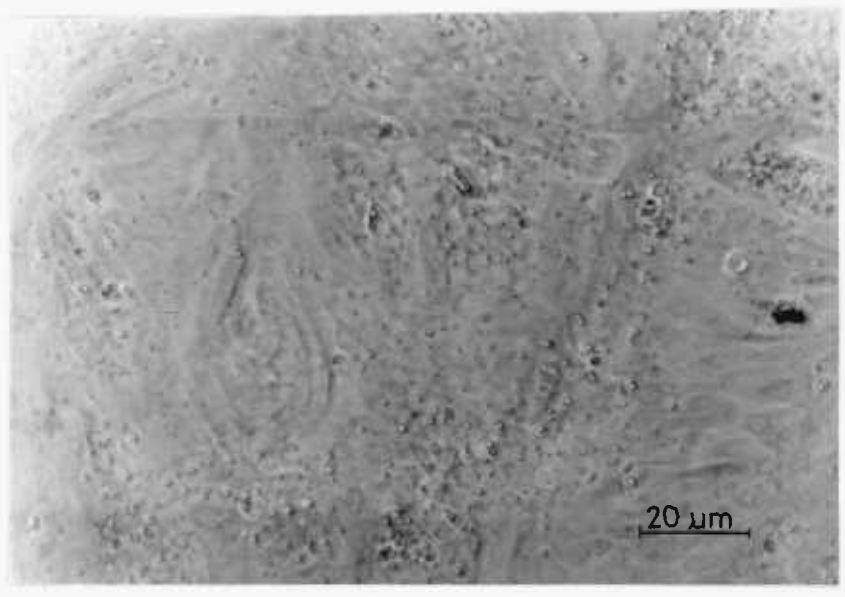
a) Control, non-parasitised cells are still confluent
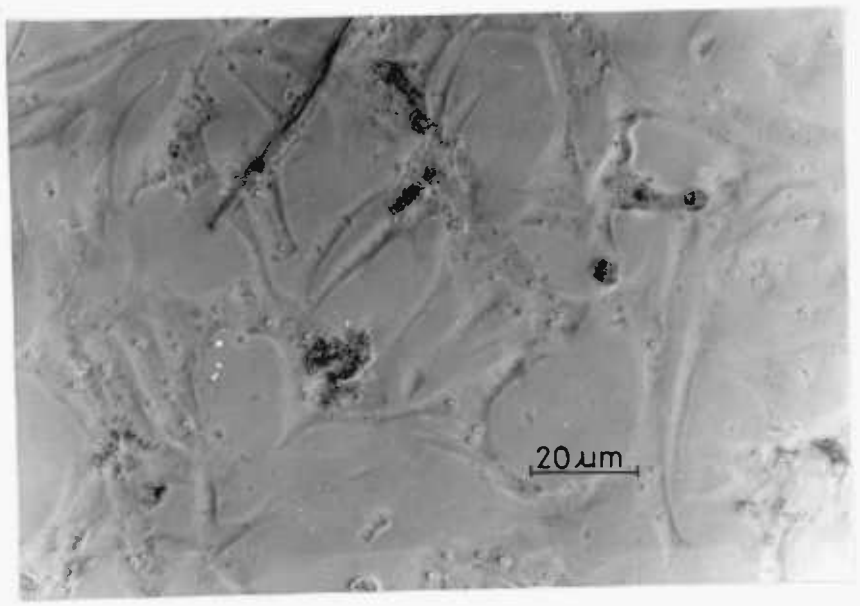
b) Experimental, parasitised cultures have developed gaps in the monolayer
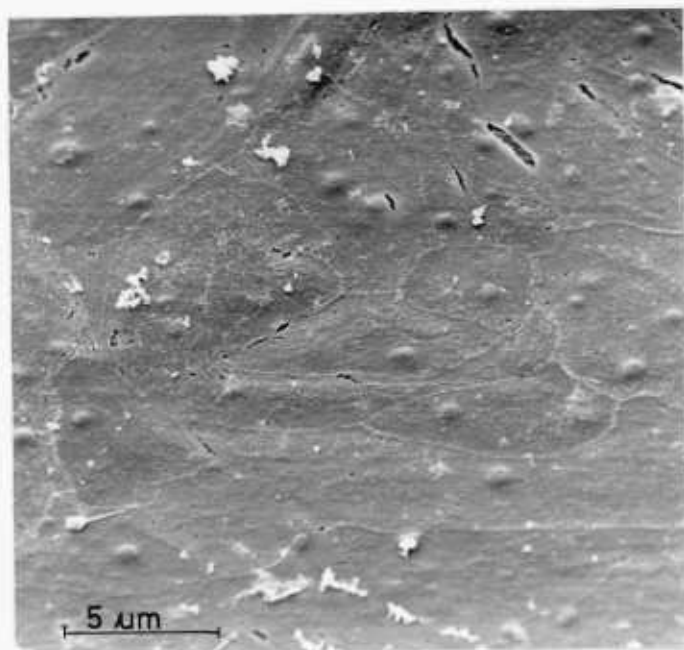
Figure 3.3.2

SEM of confluent monolayer of non-parasitised CK cells
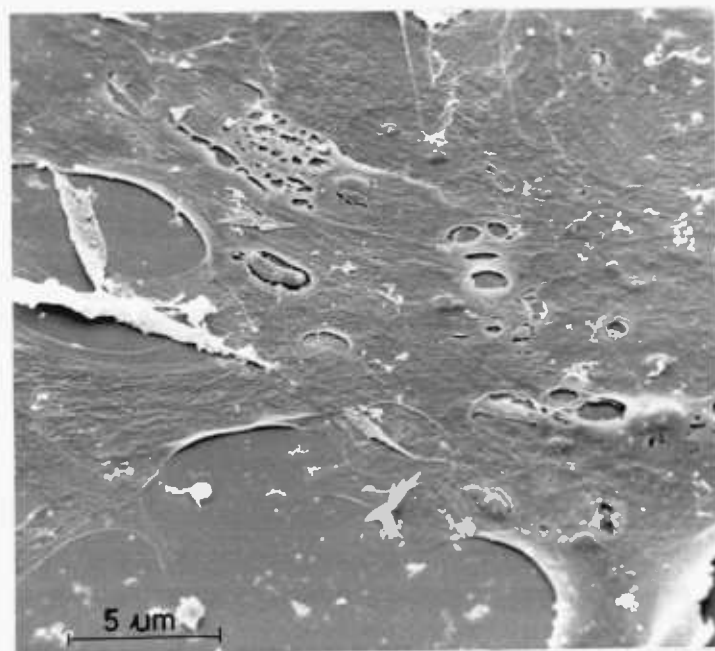
Figure 3.3.3

SEM of parasitised CK cells, 1 day p.i.
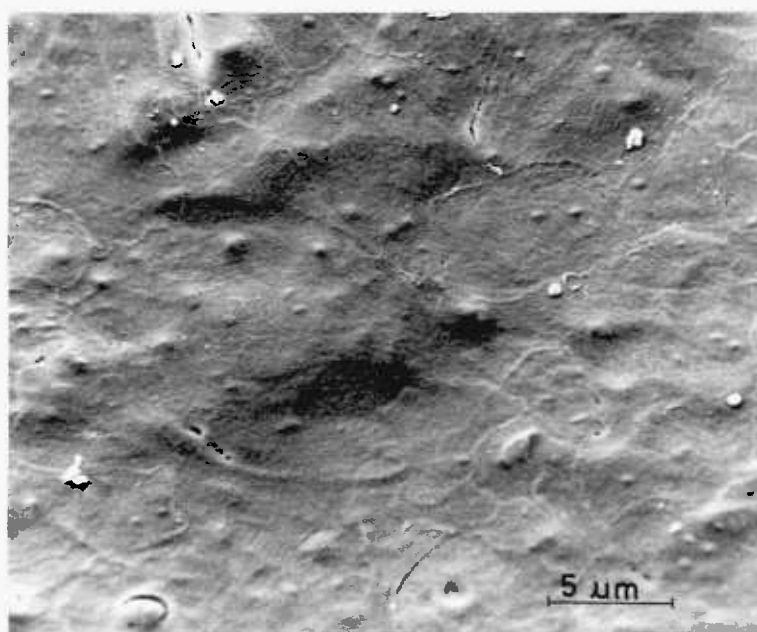
Figure 3.3.4

SEM of control, non-parasitised CK cells, 3 days p.i.

Figure 3.3.5 SEM of parasitised CK cells, 3 days p.i. Cells have rounded up and in many cases remain connected with each other only by means of cytoplasmic bridges (CB) and to the substrate by means of long retraction fibres (RF)
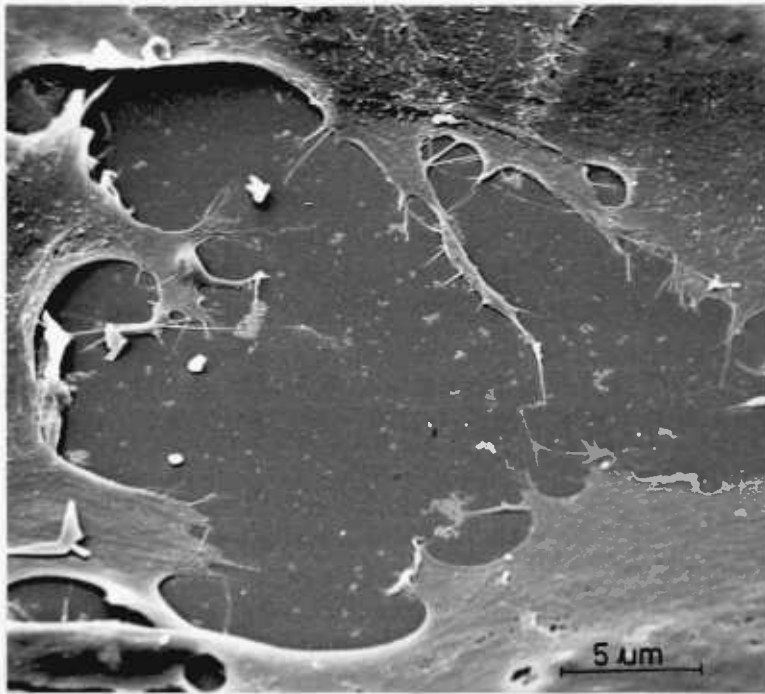
a)
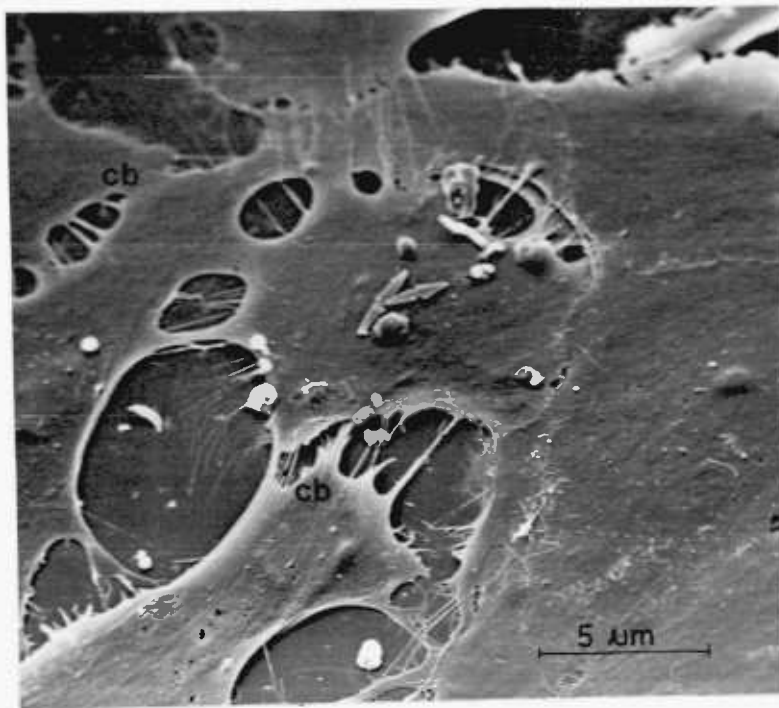
b)
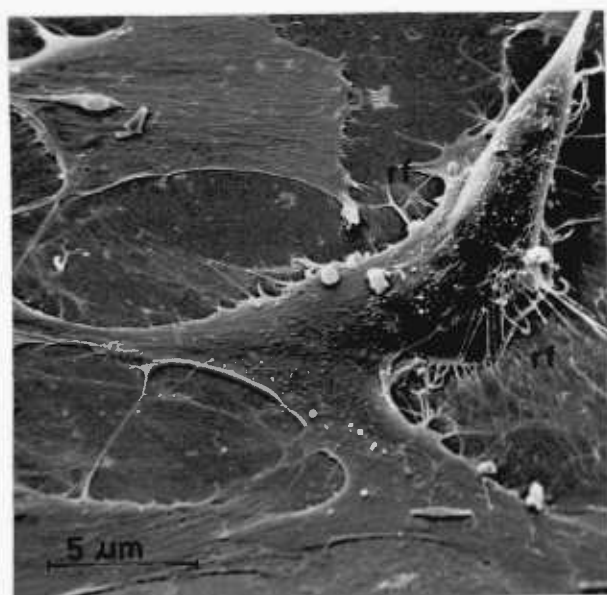
Figure 3.3.5
c)

illustrated an extreme case of this condition as it was almost totally rounded up and remained attached solely by means of the retraction fibres. Cells about to enter mitosis are known to round up prior to cell division. However, because mitotic figures were rare in cultures and the elevated cells were common in infected but not uninfected cultures, it was unlikely that they were pre-mitotic cells.

In cultures which had few confluent areas at the beginning of the experiments comparisons were made between the morphologies of single cells from parasitised and non-parasitised cultures. From non-parasitised cultures the single cells were flattened and well spread (Fig. 3.3.6a). From parasitised cultures, one day p.i., the rolled edges were again obvious and the cells appeared to be less well spread with a central cell body extending as long processes, (Fig. 3.3.6b).

The changes in cell morphology in the parasitised cultures were not limited solely to the cells which contained parasites, but affected non-parasitised cells as well. It seemed likely that parasitised cells were releasing a factor into the overlying medium which affected other cells. Alternatively, the factor could be a product of parasite metabolism. To test for the presence of this factor non-parasitised cells were grown in coculture with parasitised cells. This system allowed contact between parasitised and non-parasitised cells only via the medium. These non-parasitised cultures, when examined with the SEM after 3 days in coculture began to show changes similar to those which occurred in parasitised cells. They were difficult to distinguish from the parasitised cells, with breaks in the monolayer and cells with long processes and curled edges

Figure 3.3.6   SEM of single cells at 1 day p.i.
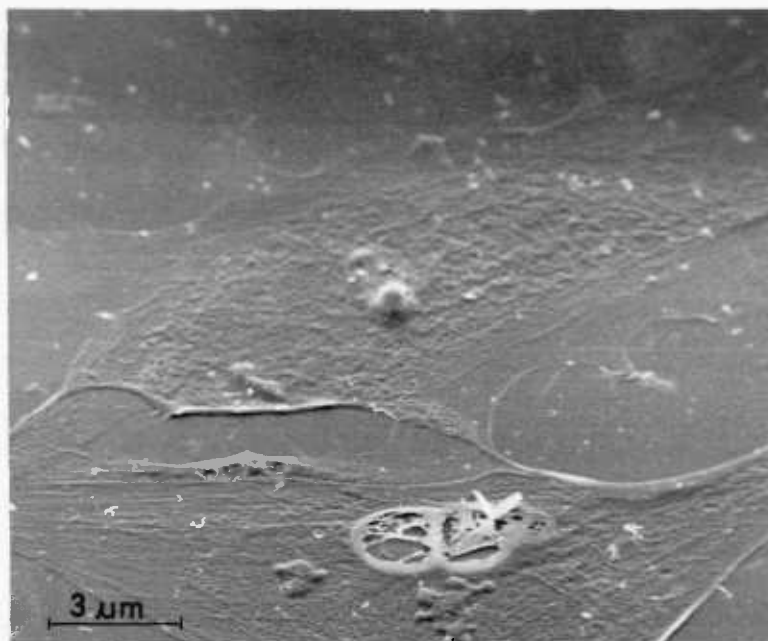
a) Non-parasitised CK cells
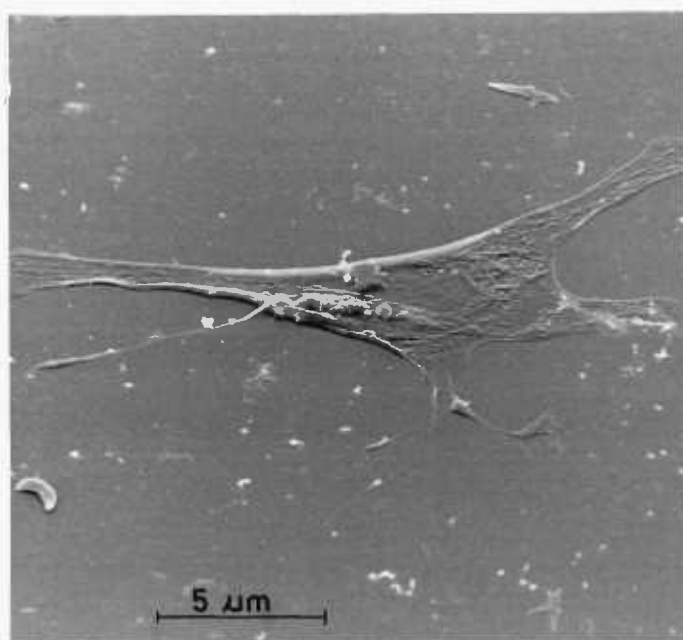
b) Parasitised CK cells

(Fig. 3.3.7). In general these changes in cocultured non-parasitised cells were very widespread, although some areas remained where the cells were still in close contact with each other (area A in Fig. 3.3.7). These changes were progressive. The results supported the theory that the parasites or parasitised cells were responsible for producing a factor which was transmissable via the medium.

It was shown that the morphological changes associated with the parasitised cultures were not due to an inoculum effect, as the parasitised cells were washed free of the inoculation medium at 12 hours, in three changes of fresh medium, prior to establishing the coculture. Furthermore the non-parasitised cocultures, which never came into contact with the inoculation medium, also showed morphological changes.

3.3.2 Adhesion Changes

3.3.2i Changes associated with the development of the parasite

The relationship between the cells and their substrate was investigated with the reflection-interference microscope technique (RIM), (Curtis 1964). By using this technique it was possible to study the separation distance between the undersurface of cells and the glass substrate on which they were growing.

CK cells in culture usually grew as discrete areas or sheets of cells, which were derived from the aggregates of the cells initially present in the trypsinised solution (2.4.2i). The RIM images of these areas showed the presence of the 3 types of cell-substrate approaches described by Izzard and Lochner (1976) for avian fibroblasts. These were focal contacts (black streaks), close contacts (grey areas) and regions of greater separation (white areas). Fig. 3.3.8 illustrates
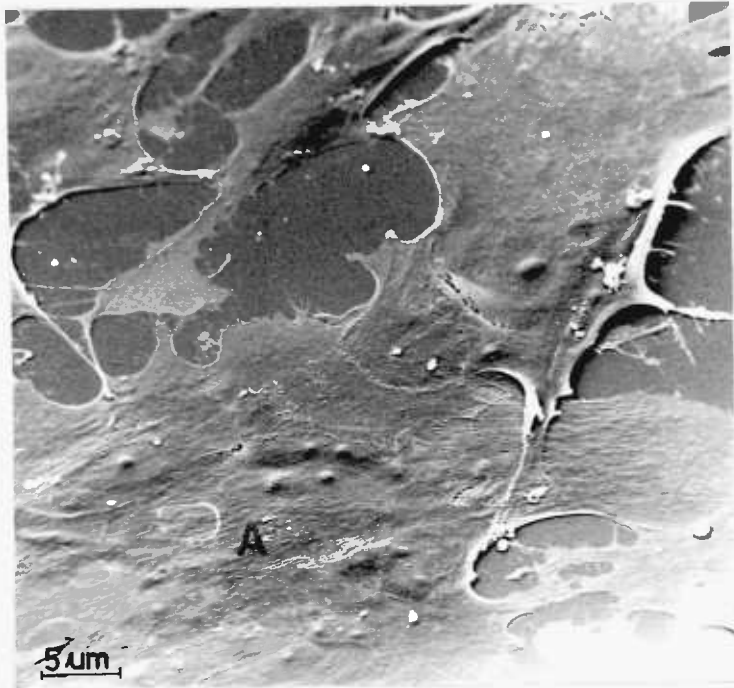
Figure 3.3.7

SEM of non-parasitised CK cells cocultured with parasitised cells for
3 days
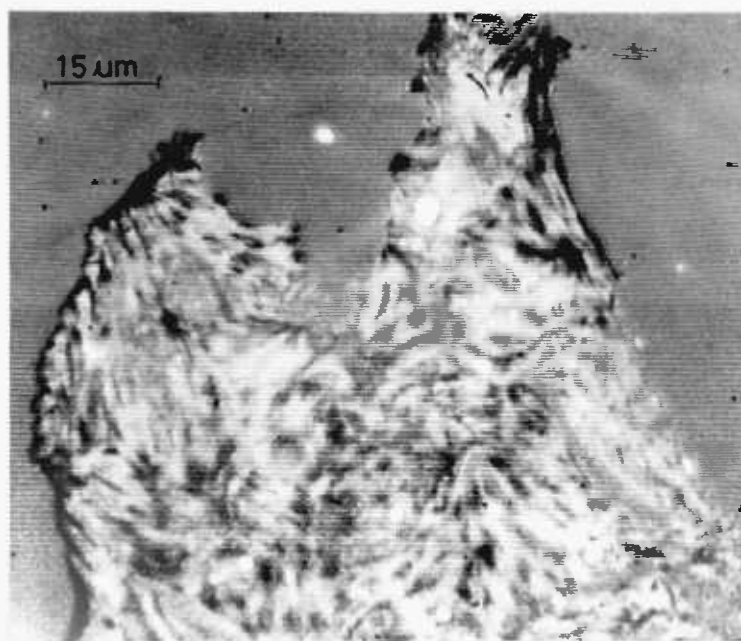
Figure 3.3.8

RIM of non-parasitised CK cells (in M199)

the edge of a group of normal, non-parasitised, CK cells. The focal contacts were areas where the cell lay at a distance of less than 15nm from the substrate (Izzard and Lochner, 1976). They formed a very regular pattern when they occurred at the edge of the cell sheet of CK cells and appeared to be lying parallel to one another and in many places perpendicular to the periphery of the cell sheet. Many areas of focal contacts were also found in the centre of the cell sheet, although the approaches here were usually less close as indicated by the more frequent occurrence of grey and white areas.

Parasitised CK cells 3 days p.i. were compared with the non-parasitised cultures of a comparable age (7 days) described above. Intracellular sporozoites and schizonts could be identified by phase contrast and the corresponding RIM image obtained without having to move the specimen. Fig. 3.3.9 shows intracellular sporozoites (arrowed) in CK cells by phase contrast illumination. Fig. 3.3.10 is the corresponding RIM image for this same area. Although dark areas were evident, they did not have the ordered arrangement of the non-parasitised group but tended to occur as haphazard areas across the sheet. The lack of image in the top left hand region (A) of the micrograph is indicative of an area of the lower surface of the sheet which was too far away from the substrate to give an interference pattern.

One of the factors involved in the formation and maintenance of adhesions is the long range physical forces. If cells are exposed to a fall in electrolyte concentration then these long range repulsive forces extend from the cell and the cell-substrate separation distance would increase (King et al 1979). If other adhesion factors, e.g.
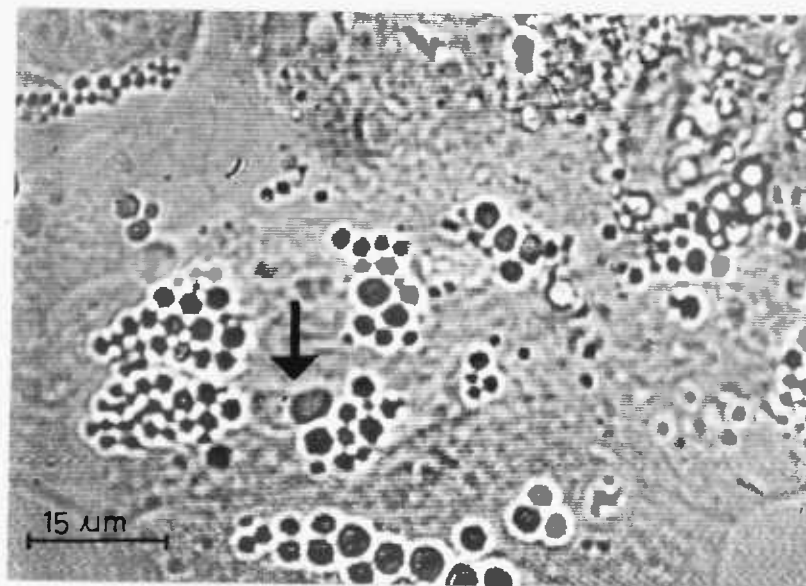
Figure 3.3.9

Parasitised CK cells as viewed with the phase contrast microscope,
3 days p.i. (Intracellular sporozoite marked with an arrow).
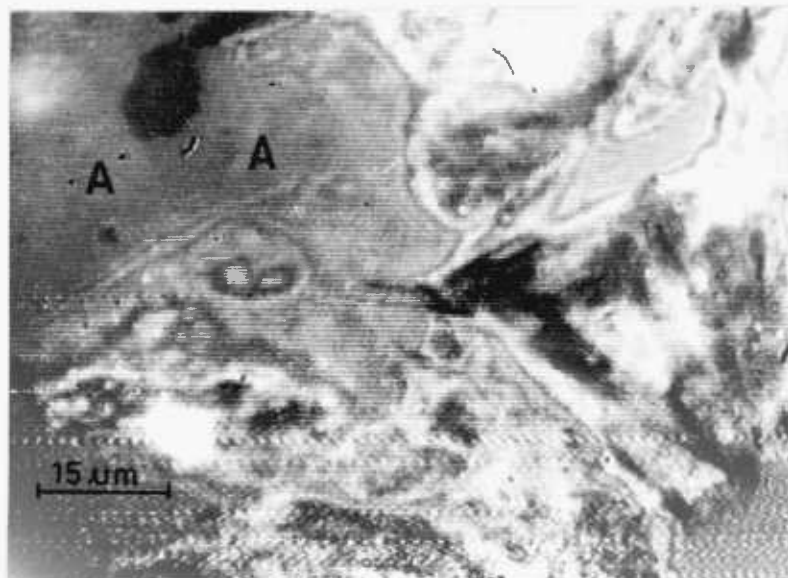
Figure 3.3.10

RIM of the same area of parasitised cells as shown in Figure 3.3.9.
(in M199)

chemical, which help to maintain a close contact between the cell and its substrate, have been lost then this increase in cell-substrate distance could ultimately result in the complete removal of the cell from its substrate.

Fig. 3.3.11 shows a cell sheet of non-parasitised CK cells with M199 in the perfusion chamber. Again the characteristic pattern of focal contacts is evident with interspersed grey areas and the white areas occupying mainly the centre of the sheet.

When the culture was perfused with a non-electrolyte solution of 0.25M sucrose (the sucrose maintained isotonicity), the undersurface of the cell sheet began to move away or lift off from the substrate as evidenced by the increasing numbers of white areas. Fig. 3.3.12 shows the appearance of the RIM image of the same cell sheet shown in Fig. 3.3.11, a few seconds following perfusion with the sucrose solution, and Fig. 3.3.13 is its RIM image at the end of perfusion. In the latter case the white areas were extensive and emphasised the focal contacts remaining at the periphery of the sheet. This sequence of events was reversible. The RIM image after flushing out the chamber with fresh M199 (Fig. 3.3.14) was comparable to the initial RIM image shown in Fig. 3.3.11.

Figs. 3.3.15 and 3.3.16 are corresponding phase contrast and RIM micrographs of another area containing a sporozoite (single arrow). Again the areas of focal contact were haphazardly arranged along the periphery of the sheet, and one edge (double arrows) was completely devoid of any focal contacts. The internal region of the cell sheet produced a white RIM image.

When this culture was perfused with the sucrose solution the RIM image showed white images or gave no image except for a few isolated
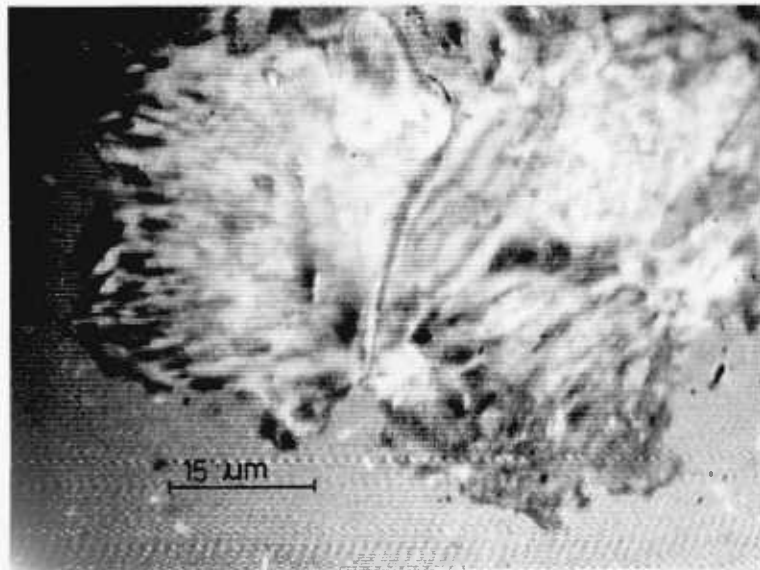
Figure 3.3.11

RIM of non-parasitised CK cells (in M199)
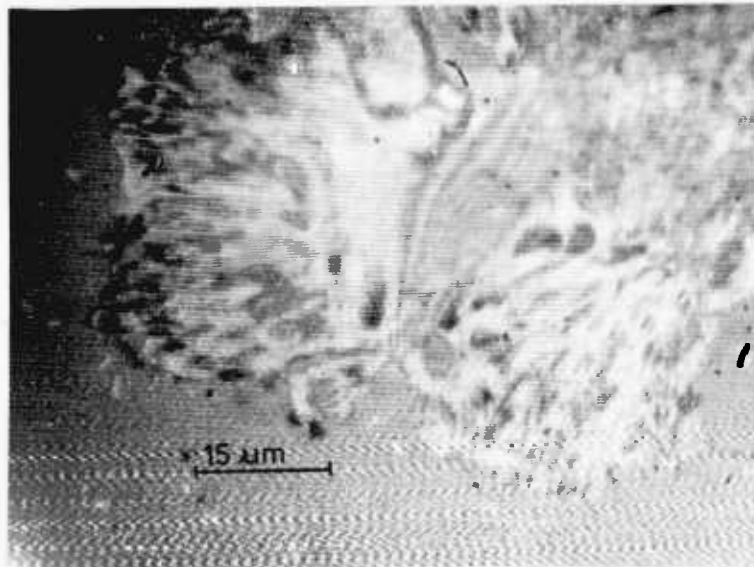
Figure 3.3.12

RIM of the same area of non-parasitised CK cells as shown in Figure 3.3.11 just after the beginning of perfusion of the culture with 0.25M sucrose
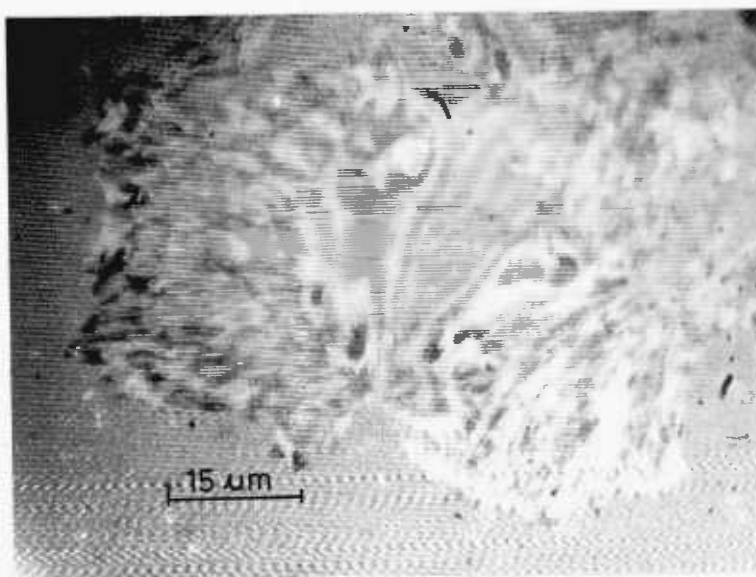
Figure 3.3.13

RIM of the same area of non-parasitised CK cells as shown in Figure 3.3.11 and Figure 3.3.12 following several minutes of perfusion with 0.25M sucrose
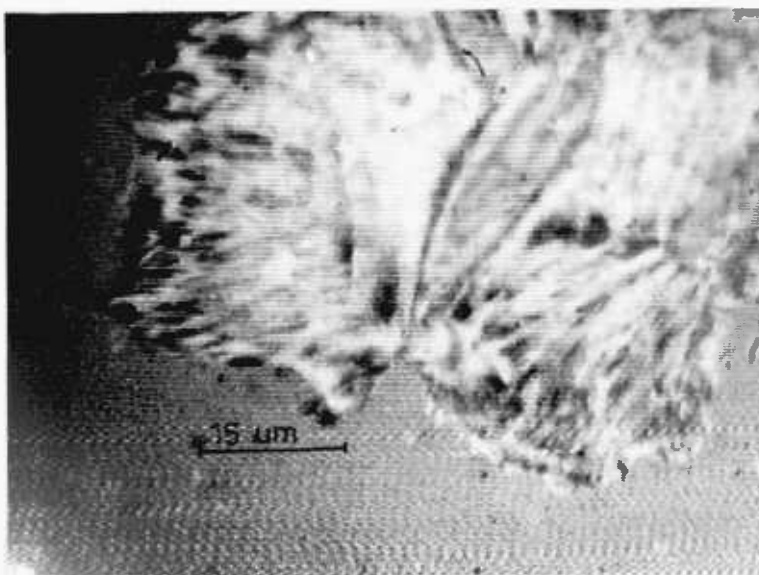
Figure 3.3.14

RIM of the same area of non-parasitised CK cells as shown in Figures 3.3.11 – 3.3.13 following the replacement of 0.25M sucrose with M199
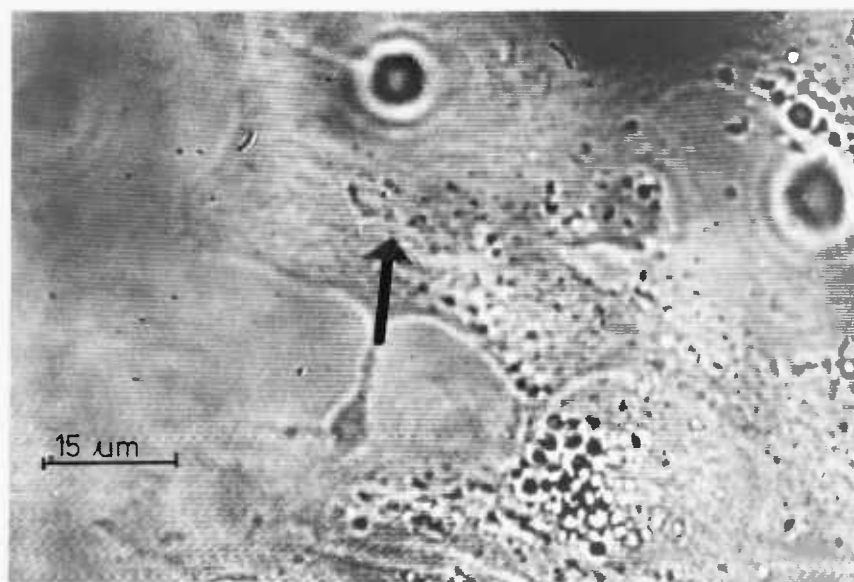
Figure 3.3.15

Parasitised CK cells as viewed with the phase contrast microscope,
3 days p.i. (Intracellular sporozoite marked with an arrow)
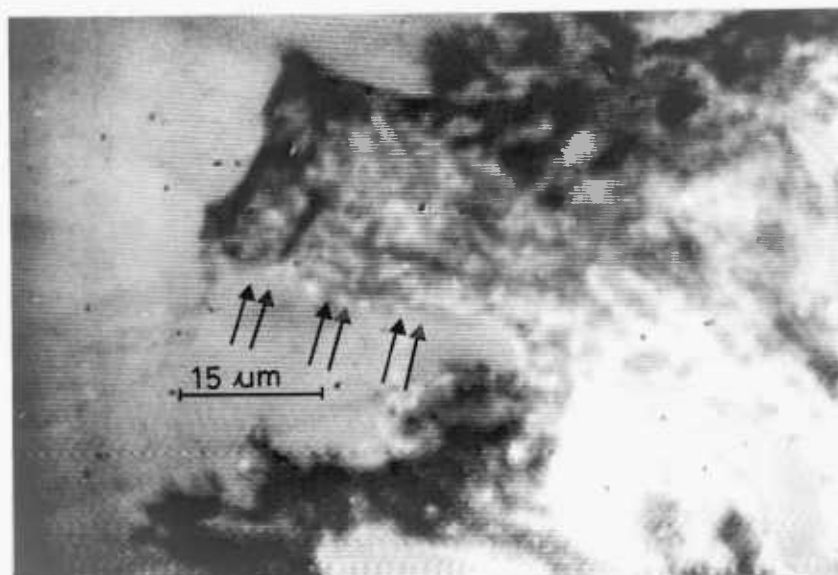
Figure 3.3.16

RIM of the same area as shown in Figure 3.3.15 (M199), the double arrows
indicate the areas devoid of contacts

patches along the edges (Fig. 3.3.17), indicating that the entire sheet had moved away from the substrate except at these edges.

A single parasitised cell (Fig. 3.3.18) gave the same response to changes in electrolyte concentration as had the larger groups of cells. The RIM image of this cell is shown in Fig. 3.3.19. Focal contacts were found along the cytoplasmic processes and close contacts occupied the centre and edges of the cell body. When perfused with sucrose the central areas of the grey images changed to a white image and then eventually produced no image at all (Figs. 3.3.20a and b, respectively). The few focal contacts persisted throughout the perfusion process.

A schizont-infected cell (Fig. 3.3.21) caused an almost complete destruction of the RIM image (Fig. 3.3.22). This was not common to all schizont-infected cells. The cell in which the schizont was growing (A) produced almost no RIM image with a small black area at one edge, and concentric white, grey, white images occupying the rest of the cell. The surrounding cells showed intermediate conditions between this extreme loss of contact and that described before in parasitised cells. For example the cell edge (B) lying above and adjacent to the schizont-infected cell was also devoid of any image, whereas the cells in position C and D still produced a few dark images.

After the perfusion of 0.25M sucrose solution, the schizont-infected cell became almost completely separated from the substrate: much of the cell produced no image at all by RIM. Surrounding cells lost most of their focal contacts and assumed a general grey image. This was 'highlighted' with many small areas of white images (Fig. 3.3.23). Re-establishment of the previous RIM image when the
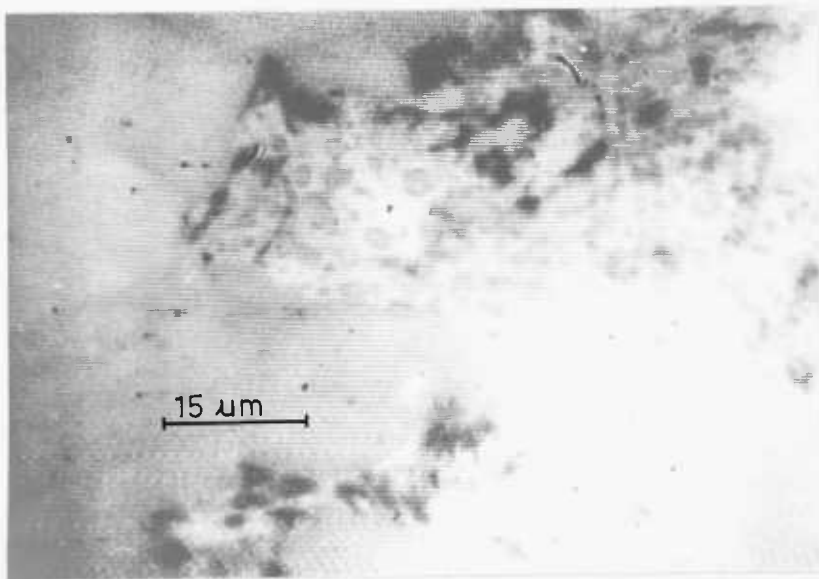
Figure 3.3.17

RIM of the same area as shown in Figure 3.3.16 following perfusion
with 0.25M sucrose
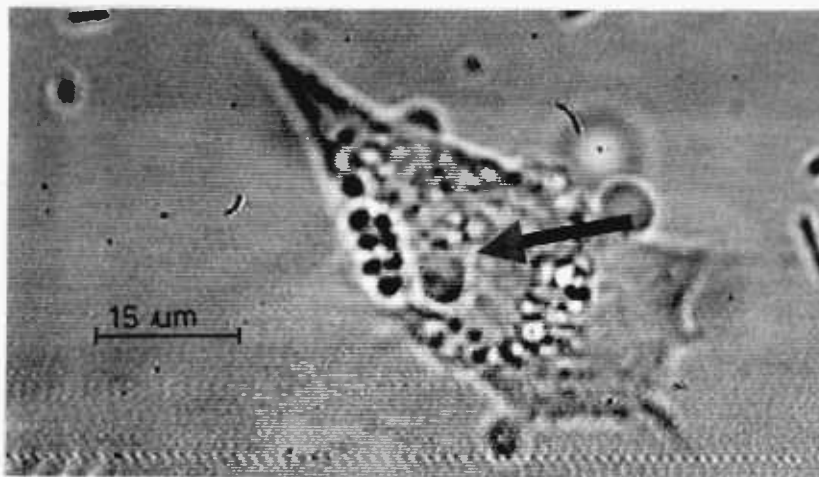
Figure 3.3.18

Parasitised, single, cell as viewed with the phase contrast microscope, (sporozoite marked with an arrow)
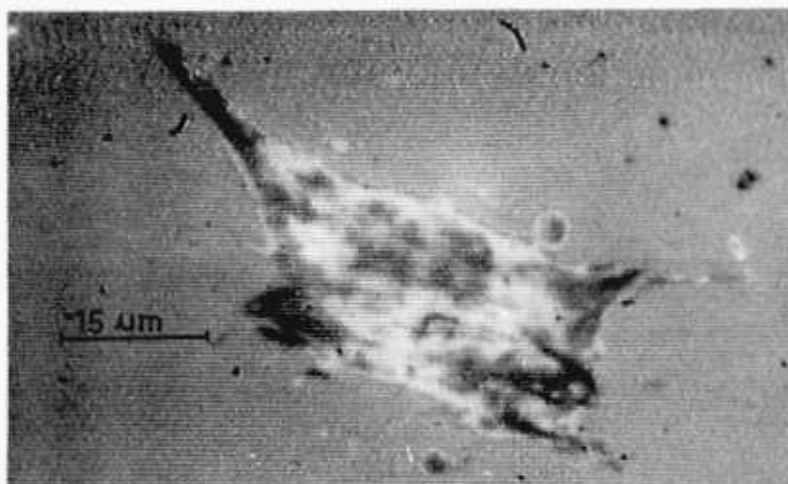
Figure 3.3.19

RIM of the same CK cell as shown in Figure 3.3.18 (in M199)

Figure 3.3.20 RIM of the cell shown in Figure 3.3.19 following
perfusion with 0.25M sucrose
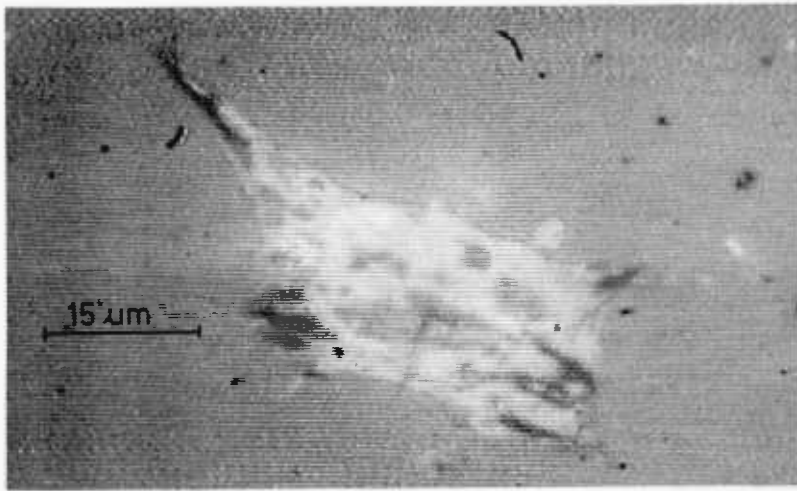
a) RIM at the beginning of perfusion
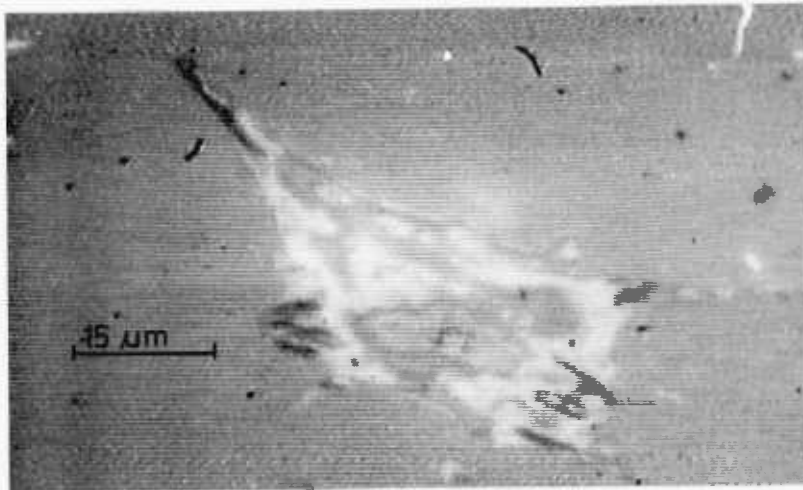
b) RIM at the end of perfusion
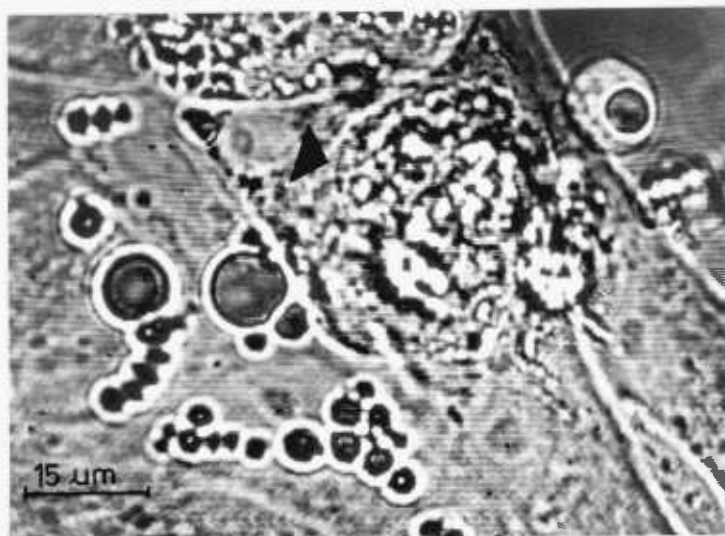
Figure 3.3.21

A schizont infected cell as viewed with the phase contrast microscope
(Schizont marked by an arrow)
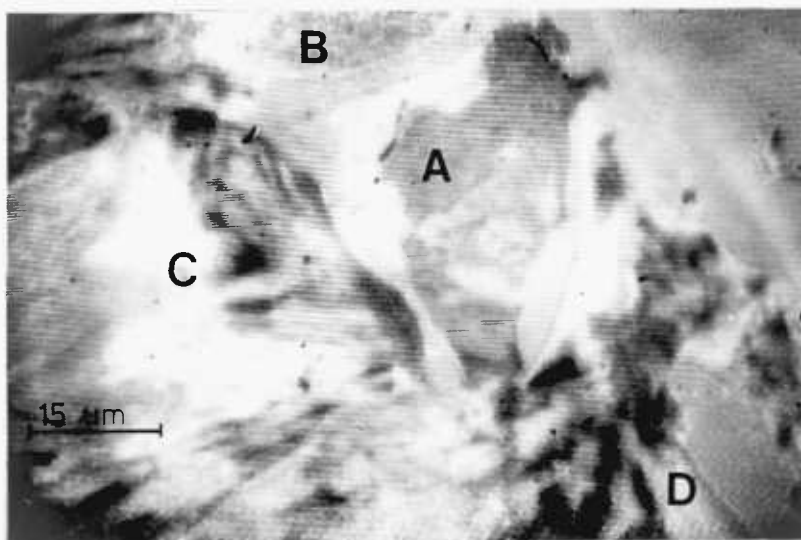
Figure 3.3.22

RIM of the schizont infected cell shown in Figure 3.3.21 (in M199).
(A- schizont; B- area devoid of contacts; C and D- areas with a few,
disorganised, focal contacts)
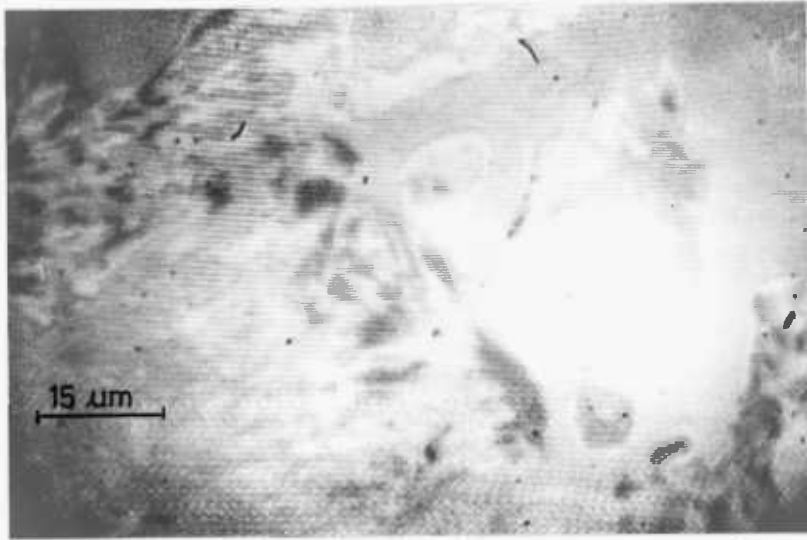
Figure 3.3.23

RIM of the schizont infected cell, shown in Figure 3.3.22, following
0.25M sucrose
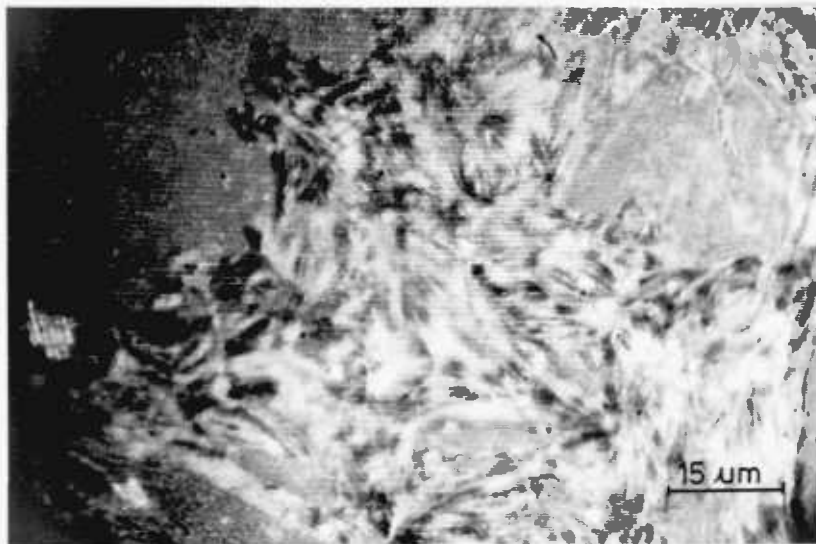
Figure 3.3.24

RIM of non-parasitised CK cells following coculture for 3 days with
parasitised cells (in M199)

culture was perfused again with M199 was not complete.

Many cells in a parasitised culture showed contact changes regardless of whether they were infected. It was therefore of interest to ascertain if there was a factor transmissable in the medium which could bring about these alterations. Non-parasitised cells were grown in coculture with parasitised cells for 72 hours and then examined with the RIM. The images produced by cocultured non-parasitised cells were identical to those produced by mono-cultured non-parasitised cells (Fig. 3.3.24). The focal contacts were arranged in an ordered fashion along the periphery and several were found scattered in the central region of the epithelial sheet.

3.3.2ii   <u>Changes associated with the invasion of the parasite</u>

CEF cultures were used to investigate alterations to the adhesion pattern of cells which occurred during invasion by <u>E. tenella</u> sporozoites. These cells were chosen because the sporozoites entered and left them in greater numbers and more rapidly than they did the CK cells in the same conditions. Furthermore a great deal of work has been done on the RIM patterns of CEF cells at many stages of their growth and so any changes to the RIM image caused by invading sporozoites could be more accurately assessed. Fig. 3.3.25 is of sporozoites invading three day old secondary CEF cultures. Intra-cellular sporozoites are marked by arrows. Fig. 3.3.26 is the corresponding RIM image produced by these cells. Focal contacts were present mainly along the edges of the cells. The 'close contact' pattern, producing a grey image, was associated with most of the remaining cell area. This pattern has been described previously as typical of such fibroblasts (King <u>et al</u> 1979) and so the invasion of
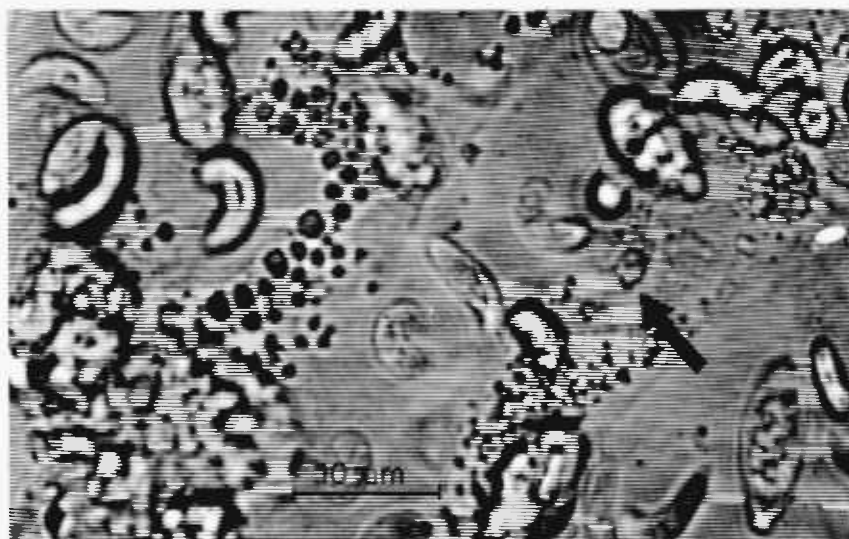
Figure 3.3.25

CEF cultures a few minutes after the addition of E. tenella sporozoites,
viewed with the phase contrast microscope. (Intracellular sporozoite
marked with an arrow)
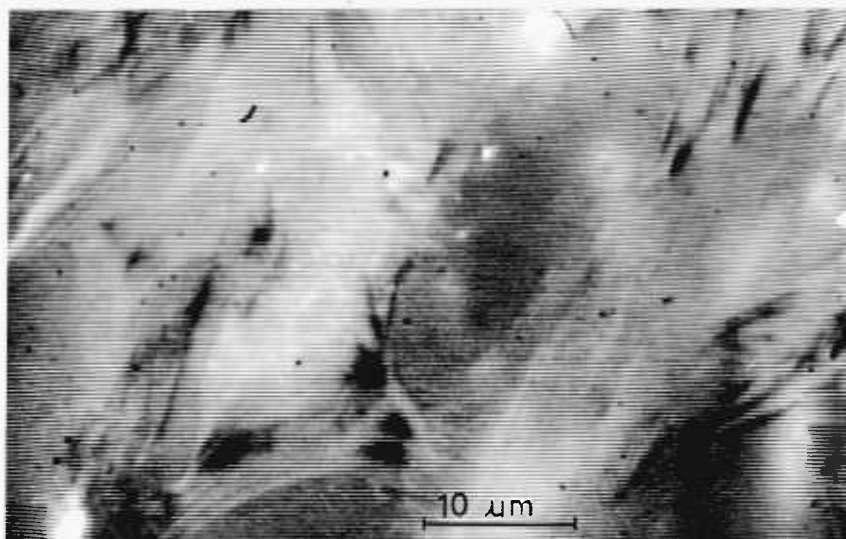
Figure 3.3.26

RIM of the same area as shown in Figure 3.3.25 (in M199)

sporozoites into cells did not appear to cause a change in their adhesions.

Sporozoites were inoculated on to newly attached cultures of human neutrophils. Most neutrophils remained uninvaded but a few intracellular sporozoites were observed, (Fig. 3.3.27). Compared with neighbouring uninfected neutrophils (double arrows) the infected neutrophil (single arrow) assumed a more rounded shape. When this group of cells was observed with the RIM the images were markedly different (Fig. 3.3.28). Uninfected neutrophils gave a characteristic area of focal contact which marked the cell body. Neutrophils leave tracks on the substrate as they move and these produce a grey image in the RIM. The infected neutrophil differed from the normal in that the cell body showed no focal contacts and there were no associated tracks. The cell had ceased to move once invaded.

3.3.3 Uptake of ^{3}H-thymidine by parasitised and non-parasitised cells in mono- and co-culture

Evidence from SEM studies of CK cultures (Section 3.3.1) had indicated the presence of a substance or substances in the overlying medium of parasitised cultures which could affect the morphology of non-parasitised cells in parasitised cultures or in co-cultures. A more quantifiable system was chosen to ascertain if the factor(s) involved have more widespread effects. Coccidia are known to stimulate their host cells to take up excess thymidine in vivo and in vitro (Browning et al 1976). This response is not complicated in experimental investigations because of the inability of the parasites themselves to take up an exogenous supply of thymidine (Ouellette, Strout and McDougald 1973). The ability of the parasites, directly or

Figure 3.3.27

Human neutrophils with invading E. tenella sporozoites, as seen with the
phase contrast microscope.
Double arrow: uninfected neutrophils
Single arrow: infected neutrophil
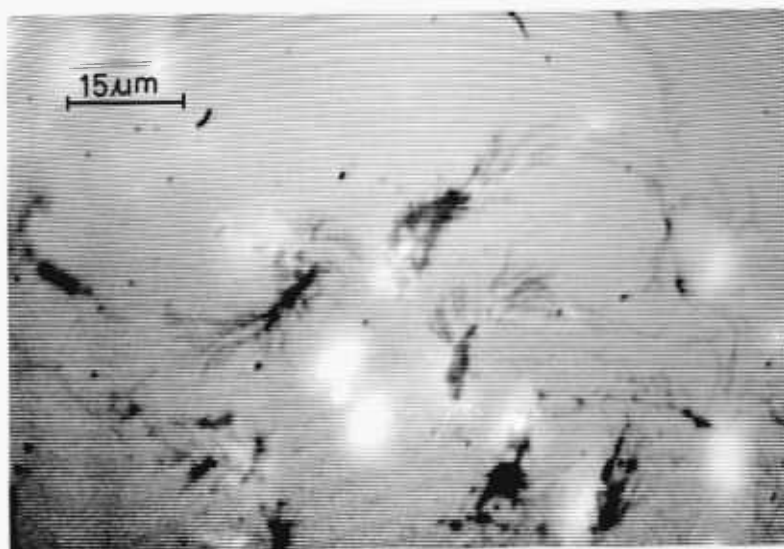
Figure 3.3.28

RIM of the same neutrophils as seen in Figure 3.3.27 (in M199)

through their host cells, to stimulate additional uptake of thymidine

by non-parasitised cells was investigated using the technique of

coculture previously described (2.4.6 and 3.3.1). Cultures were

pulsed at various intervals for 1 hour with a solution of HBSS

containing ^{3}H-thymidine at 2µCi/ml, and the coverslips processed for

autoradiography as described in section 2.7.2. Thymidine uptake was

measured by counting the percentage of labelled nuclei and the

results for the three types of cultures are given in Fig. 3.3.29.

Autoradiography was used to analyse the uptake as it provided an

answer to whether any observed stimulation was a qualitative or

quantitative effect; such information could not have been obtained

from scintillation counts.

The background level of incorporation was low, with just over

3% of the nuclei being labelled. This fell unaccountably in the

parasitised culture at 12 hours p.i. However, after 12 hours

parasitised and non-parasitised monocultures showed increased

labelling, reaching a maximum level at 20 hours p.i. The parasitised

cultures showed a percentage labelling of the nuclei of 8.35 $\pm$ 0.40,

and the non-parasitised 4.73 $\pm$ 0.36. When the non-parasitised cells

were placed in coculture they showed a percentage labelling of their

nuclei of 7.47 $\pm$ 0.39, intermediate between these two values,

(significant, $p < 0.001$, Student's t test). This uptake reached a peak

at 20 hours p.i., or 8 hours from the beginning of coculture. Most

parasites had invaded by twelve hours following inoculation (Fig. 3.2.1)

and this, together with the fact that parasitised cultures were washed

free of all non-invaded sporozoites at 12 hours, indicated that the

stimulation of thymidine uptake in parasitised and cocultured non-

parasitised cultures was an effect of the invaded and not the free

Fig. 3.3.29. Uptake of ^{3}H Thymidine: effect on host cell response when non-parasitised cells are cocultured with parasitised cells.

x——x non-parasitised monocultures

□– –□ non-parasited co-cultures

o····o parasitised monocultures

COCULTURE

% labelled nuclei
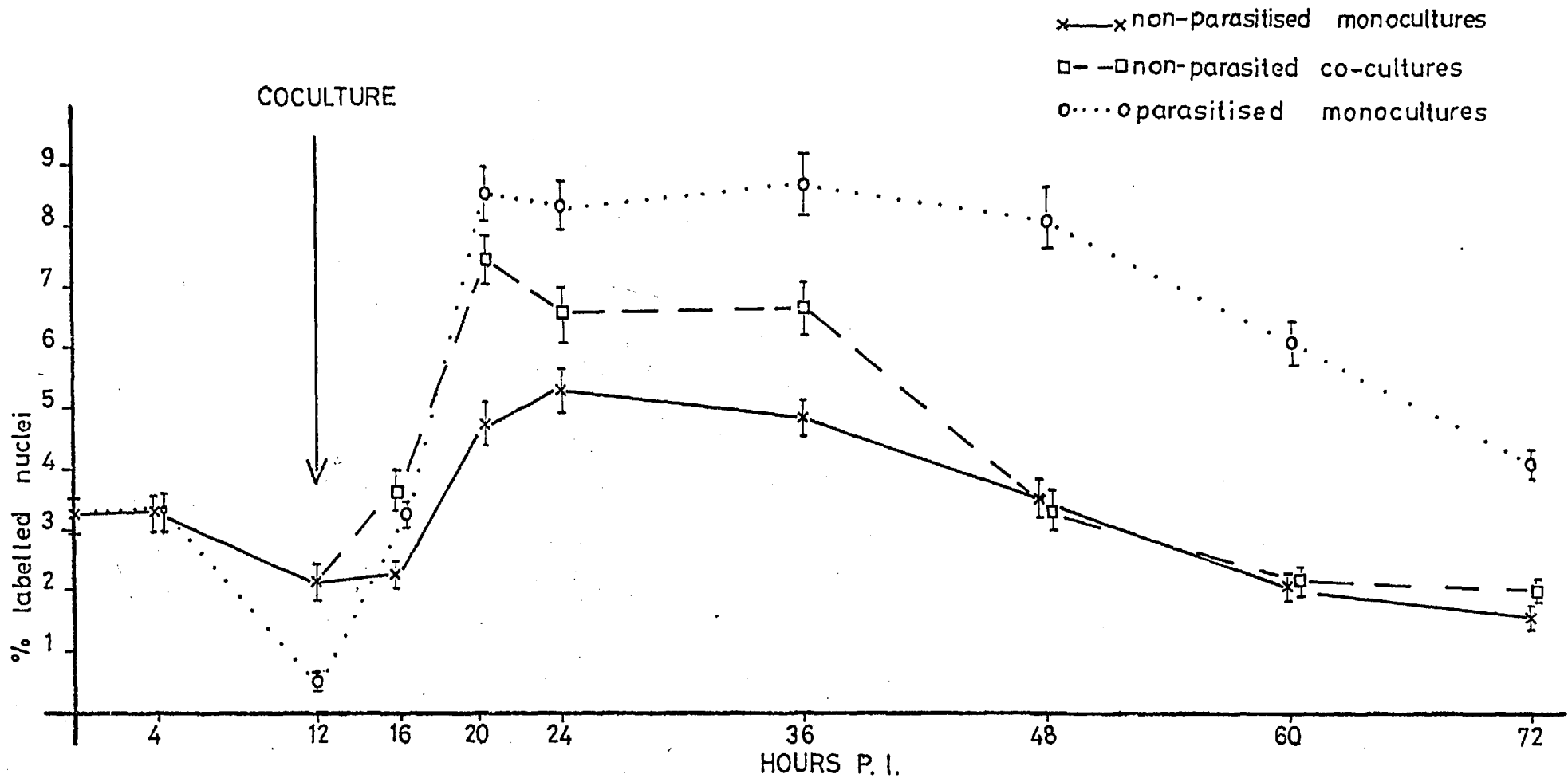
HOURS P. I.

parasites.

At 48 hours the number of labelled nuclei began to decline in
the parasitised cultures. This decline became noticeable at an
earlier stage in both the non-parasitised cultures (at 36 hours p.i.) and
by 48 hours p.i. both these had returned to the background level.

The ratio of labelled to non-labelled nuclei was compared between
non-parasitised cells growing in coculture and those cells from the
parasitised culture (hereafter referred to as the mixed culture) which
were in fact non-parasitised (Fig. 3.3.30). The results indicated
there was no real difference between the thymidine uptake of either of
the populations, suggesting that non-parasitised cells growing amongst
the parasitised cells in a mixed culture were receiving no more
stimulation than those in touch with the parasitised cells only via the
medium.

However, the ratio of labelled to non-labelled cells was greater
in the parasitised cells than the non-parasitised cells from the
mixed culture (Fig. 3.3.31). Consequently when the ratios of
parasitised to non-parasitised cells were compared (Fig. 3.3.32) there
was a big difference between that of the non-labelled and labelled
cells from this culture. These differences were only evident after
24 hours p.i., 4 hours later than the peak of ^{3}H-thymidine uptake.

3.3.4 Agglutinability with Concanavalin A (Con A)

The lectin Con A was used in agglutination assays of cells from
parasitised and non-parasitised cultures. In a preliminary experiment
Con A was used at 100μg/ml and the two cultures were assessed for
agglutination on a scale of + to +++++. This was performed 2 days
after inoculation of parasites on to the cultures, and results are

Fig.3.3.30. Ratio of L/NL nuclei in two non-parasitised cell populations.

$\frac{L}{NL}$

L_ labelled nuclei   •——• coculture

NL_ non-labelled nuclei   o---o mixed culture
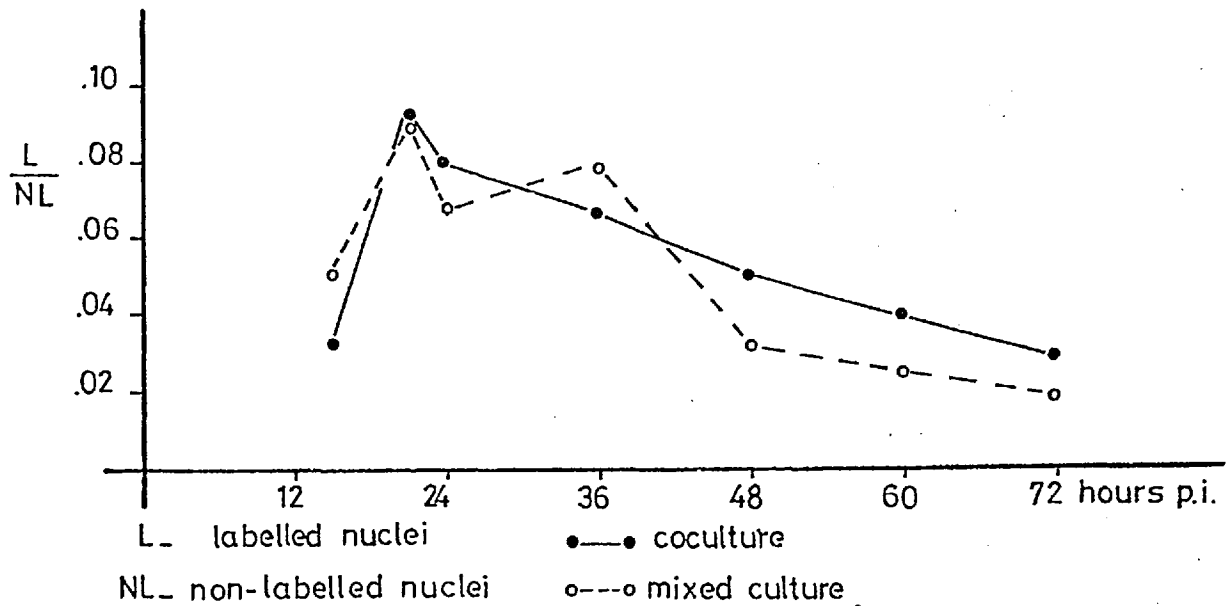
Fig.3.3.31. Ratio of L/NL nuclei for two cell types in a mixed culture.

$\frac{L}{NL}$

L — labelled nuclei   •——• parasitised cells

NL — non-labelled nuclei   o--o non-parasitised cells
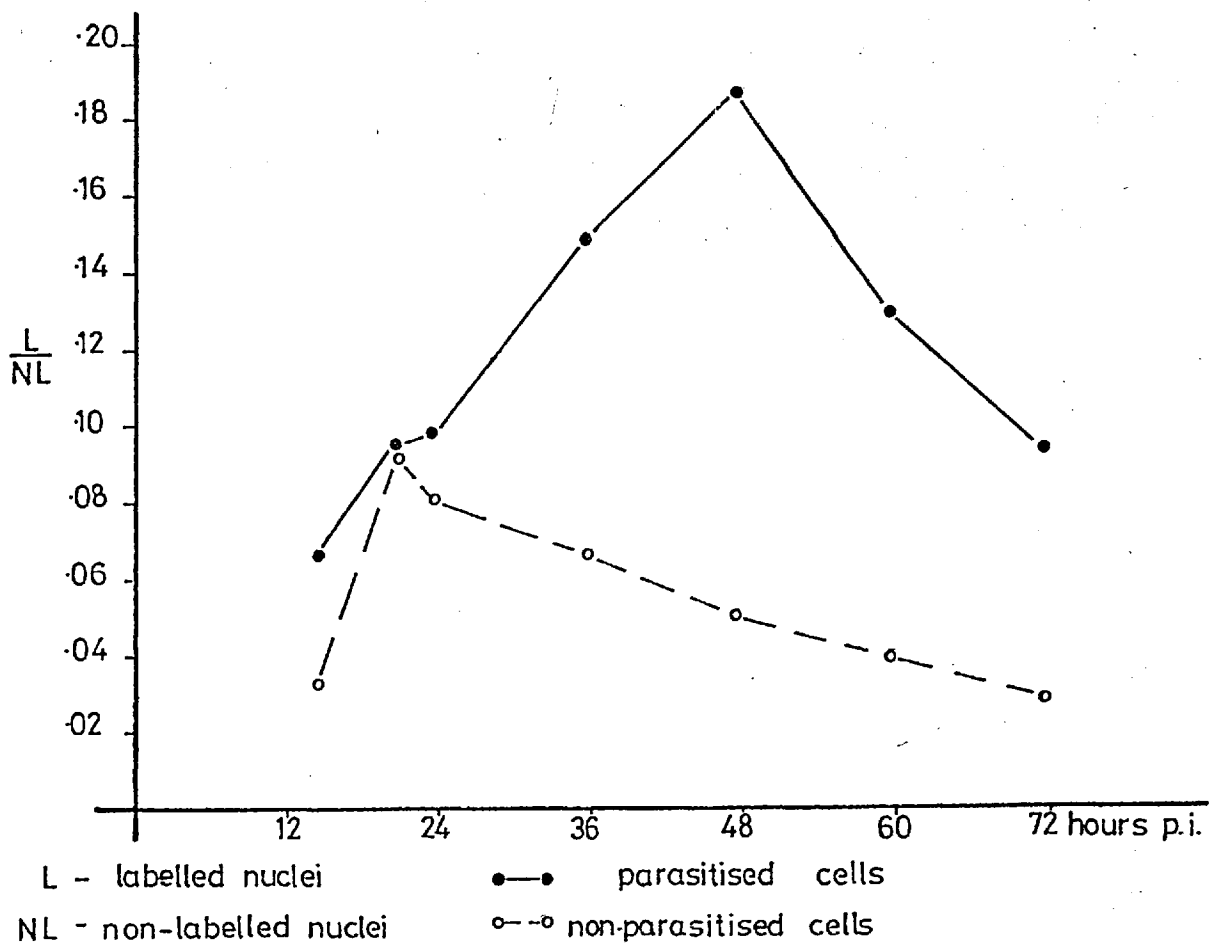
Fig. 3. 3. 32. Ratio of P/NP in labelled and non-labelled cells.
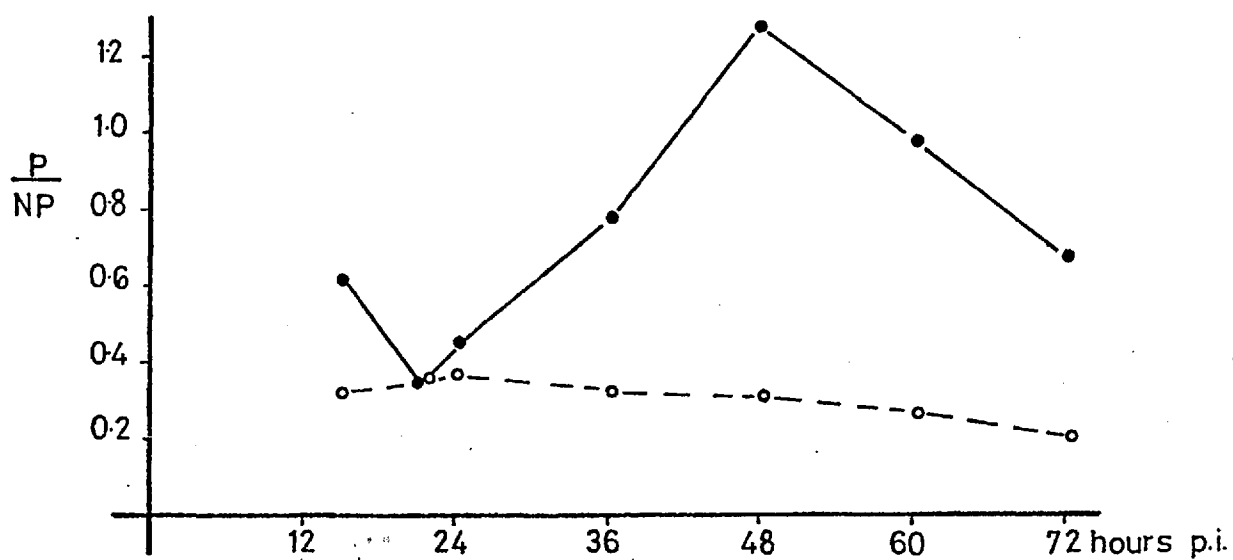
NP — non-parasitised cells   P — parasitised cells

●——● labelled nuclei   o— —o non-labelled nuclei

shown in Table 3.3.1. DNAase was added to all groups as DNA leakage which may occur during the suspension process can cause cells to stick, thereby obscuring true agglutination (Schnebli, 1976).

Table 3.3.1 Agglutinability of CK cells with Con A

Con A 100µg/ml	DNAase 1mg/ml	Parasites	Agglutination Score
✓	✓	-	++
-	✓	-	++
✓	✓	✓	++++
-	✓	✓	+

Although the background score was ++, the presence of parasites in CK cells for 2 days increased the agglutination score to ++++. As the score for cells without Con A was also ++ it suggested that this was a value for the background aggregation of the cells and therefore did not represent any real agglutination.

Agglutinability of the parasitised cultures was then assessed with serial dilutions of Con A from 100 µg/ml to 0.1µg/ml, all solutions contained DNAase at 1mg/ml. The results are shown in Table 3.3.2.

Table 3.3.2 Agglutinability of parasitised cultures with serial

dilutions of Con A

Concentration of Con A (µg/ml)	Parasites	Agglutination
100	-	++
100	✓	+++++
10	✓	++
1	✓	0
0.1	✓	+
-	✓	+++

Background aggregation in these cultures one day p.i. was +++.

However, with Con A at 100µg/ml the agglutinability was +++++ which

was higher therefore than the background. At concentrations below

100µg/ml the parasitised cells did not agglutinate.with Con A.

Fig. 3.3.33 and 3.3.34 show the appearance of non-parasitised and

parasitised cultures, respectively, following a 30 minute incubation

with Con A at 100 µg/ml. The parasitised cultures are noticeably more

clumped than the non-parasitised cultures which are composed mainly of

single cells.

A comparison was made of the agglutination with Con A of

parasitised and non-parasitised cells, which had been fixed prior to

the agglutination assay, in a 2% glutaraldehyde solution for 30

minutes at 4°C. The results given in Table 3.3.3.were obtained by

counting the number of single cells in every 100. This method was used

because the high background made the scoring of clumps by eye rather

risky.
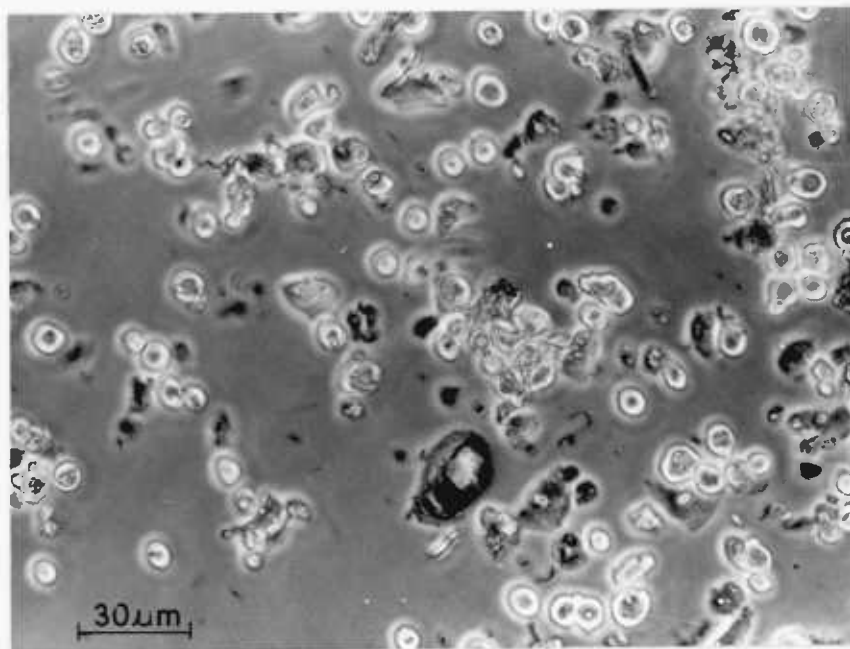
Figure 3.3.33

The appearance of a suspension of non-parasitised CK cells after 30 minutes incubation with Con A
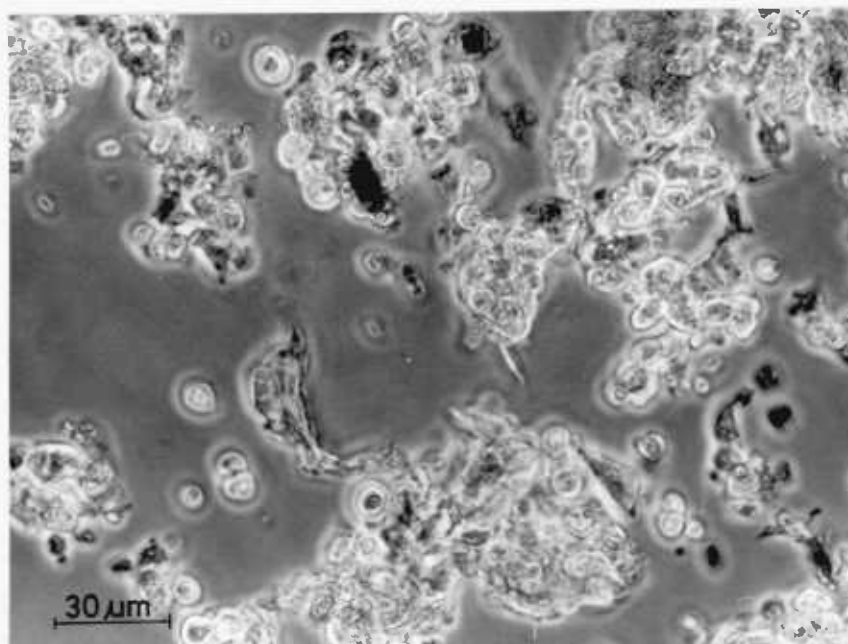
Figure 3.3.34

The appearance of a suspension of parasitised CK cells after 30 minutes incubation with Con A

Table 3.3.3 Agglutinability of fixed cells with Con A

Group	Con A 100μg/ml	DNA-ase/mg/ml	Parasite	2% glutaraldehyde	% single cells
A	✓	✓	-	-	58.4 ± 3.96
B	-	✓	-	-	65.0 ± 2.75
C	✓	✓	✓	-	41.7 ± 3.49
D	-	✓	✓	-	61.3 ± 2.52
E	✓	✓	-	✓	53.6 ± 3.93
F	-	✓	-	✓	71.1 ± 1.63
G	✓	✓	✓	✓	50.1 ± 3.50
H	-	✓	✓	✓	74.1 ± 2.97

The data from this experiment were analysed using a three way analysis of variance. This statistical test indicated that the presence of parasites and Con A caused a significant decrease in the number of single cells at the end of the incubation period (F test, $p < 0.05$) when compared with the non-parasitised/Con A group. However, the fixation of parasitised cells with glutaraldehyde caused a significant decrease in agglutinability (F test, $p < 0.05$) of these cells with Con A, and there was no longer a significant difference between the agglutinability of parasitised and non-parasitised cells ($p > 0.05$).

3.3.5 Loss of labelled glycoproteins from the surface of cultured cells following parasitisation with E. tenella

Glycoproteins were labelled by growing cells in medium containing ^3H-l-fucose over a period of 72 hours. Changes in the surface glycoproteins of the cells were followed by analysing alterations in the amount of radioactivity associated with the cultures, and by monitoring

the appearance of radioactivity in the medium overlying the cells.
In the following record of the results the former is referred to as
the coverslip preparation and the latter as the supernatant prepar-
ation. Initially the changes were monitored over the first 48 hours
following inoculation of the cultures with sporozoites. This
inoculation was coincident with the removal of the cultures from the
radiolabelled solution to the non-labelled solution. The results are
shown graphically for the coverslip and supernatant preparations in
Fig. 3.3.35. In both groups there was a substantial loss in the
radioactivity associated with the coverslip preparations in the first
24 hours. It amounted to a fall in radioactivity of approximately
64%. After 24 hours the fall in radioactivity levelled off in both
cultures (Fig. 3.3.35a). With the supernatant preparations, the
associated radioactivity rose slowly over the first 24 hours p.i. the
increase being the same in both parasitised and non-parasitised cultures.
By 48 hours p.i. there was more ^{3}H-1-fucose in the supernatant of
parasitised cultures (Fig. 3.3.35b). Although the increase in radio-
activity seen in the supernatants was not as great as the loss from the
coverslips, the two results cannot be directly compared as the method
of preparation of coverslips and supernatants was not identical and
could have resulted in differing amounts of autoquenching.

.The greater gain of radioactivity in the supernatant of
parasitised cultures could have been due to either i) parasitised cells
releasing more glycopeptides into the medium, possibly as a result of
proteolytic activity or ii) the loss of whole cells or cell fragments.

The experiment was repeated over a 5 day period, p.i. Loss of
radioactivity from the coverslips (Fig. 3.3.36) followed a similar

Fig.3.3.35 Changes in the radioactivity, associated with ^{3}H-l-fucose, following the inoculation of CK cells with E.tenella sporozoites.

a)Radioactivity associated with the cells.

b)Radioactivity associated with the supernatants.

o--o non-parasitised cultures
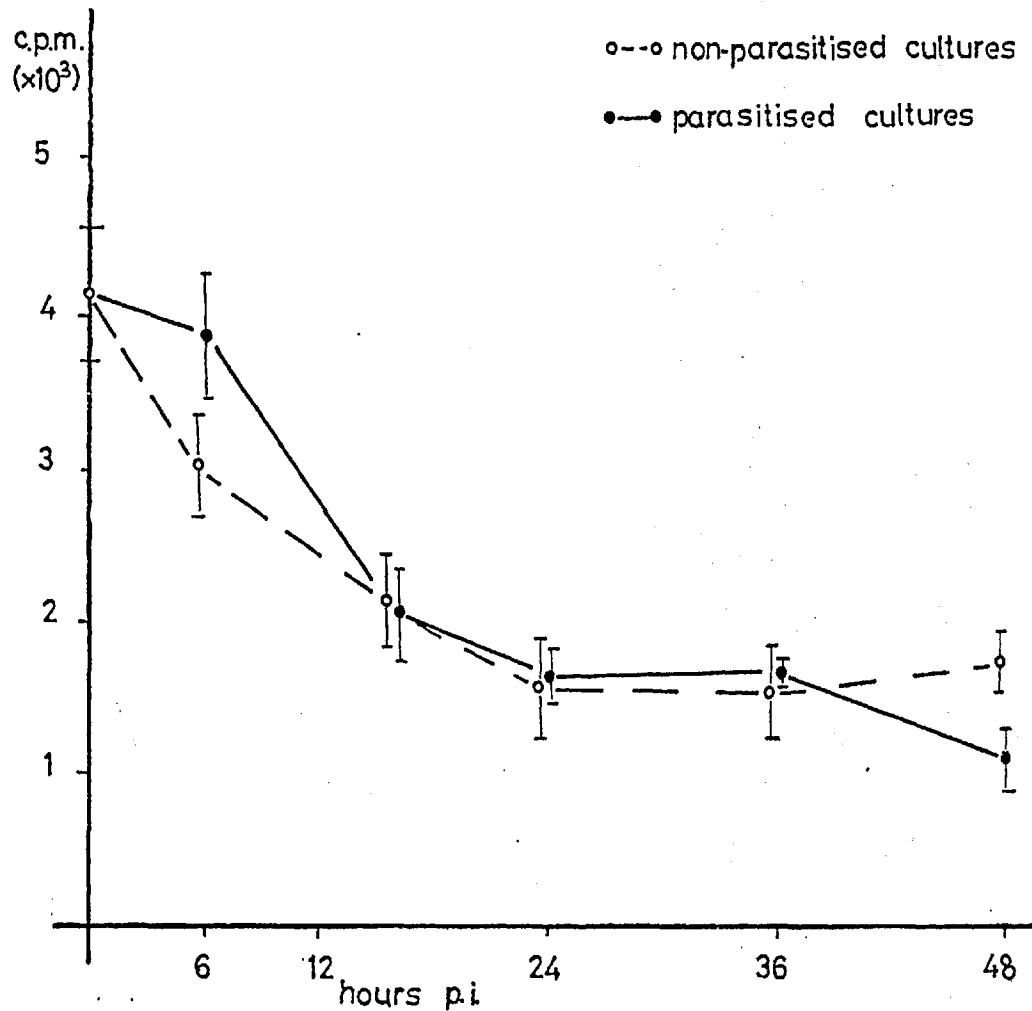
•—• parasitised cultures
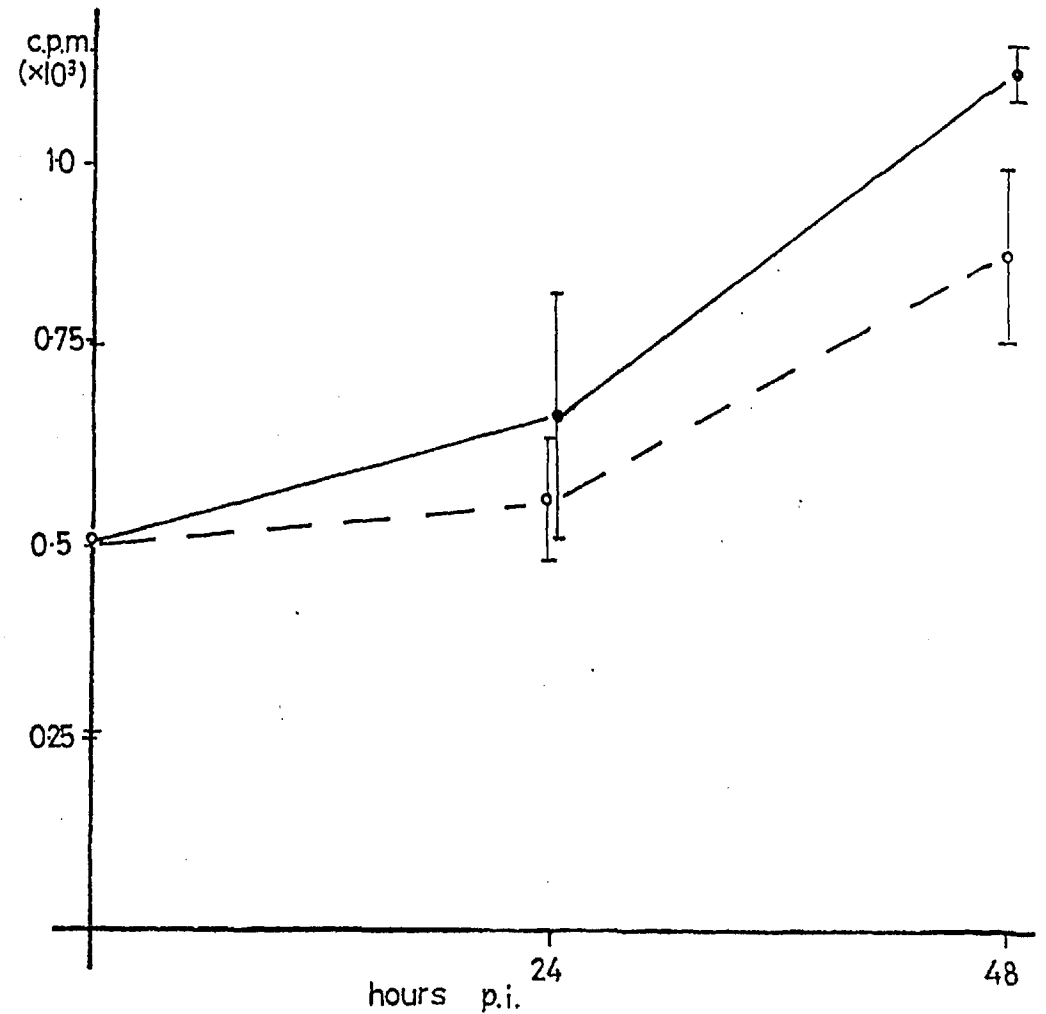
Fig.3.3.36. <u>Loss of radioactivity from CK cells labelled</u> <u>with ^{3}H-l-fucose following inoculation</u> <u>with sporozoites of E.tenella.</u>

c.p.m. ($\times 10^2$)

o--o non- parasitised cultures

•—• parasitised cultures
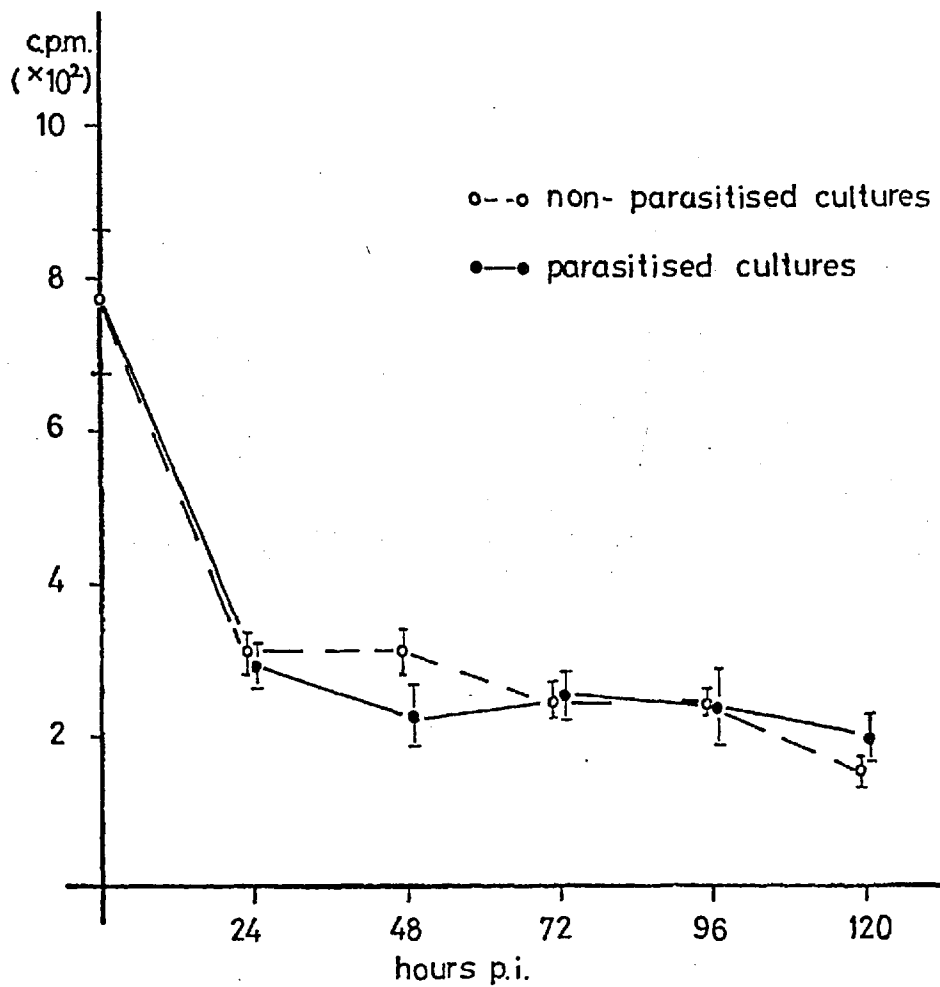
hours p.i.

biphasic pattern to that observed before (Fig. 3.3.35a). There was

a great loss over the first 24 hours p.i., and then a slow decline

in radioactivity over the following 4 days. The pattern of loss was

identical in both cultures.

The difference in radioactivity in the supernatants became

increasingly greater over the 5 day period, so that by day 5 p.i.

there was nearly twice as much radioactivity associated with the

supernatant from parasitised cultures compared with that from non-

parasitised cultures (Fig. 3.3.37). However, spinning down the

supernatant before the TCA was added removed nearly all radioactivity

from the supernatants of both cultures. This suggested that it had

been associated with cell membrane fragments, which were present in

greater numbers in the supernatants from parasitised cultures, and

was not due to small glycopeptide chains released into the supernatants

from the cell surface.

Fig. 3. 3. 37.

Gain of radioactivity by the supernatants of CK cultures labelled with ^{3}H-l-fucose and subsequently inoculated with sporozoites of E. tenella.

o non-parasitised cultures
• parasitised cultures
—— untreated supernatants
– – – centrifuged supernatants

Hours p.i.
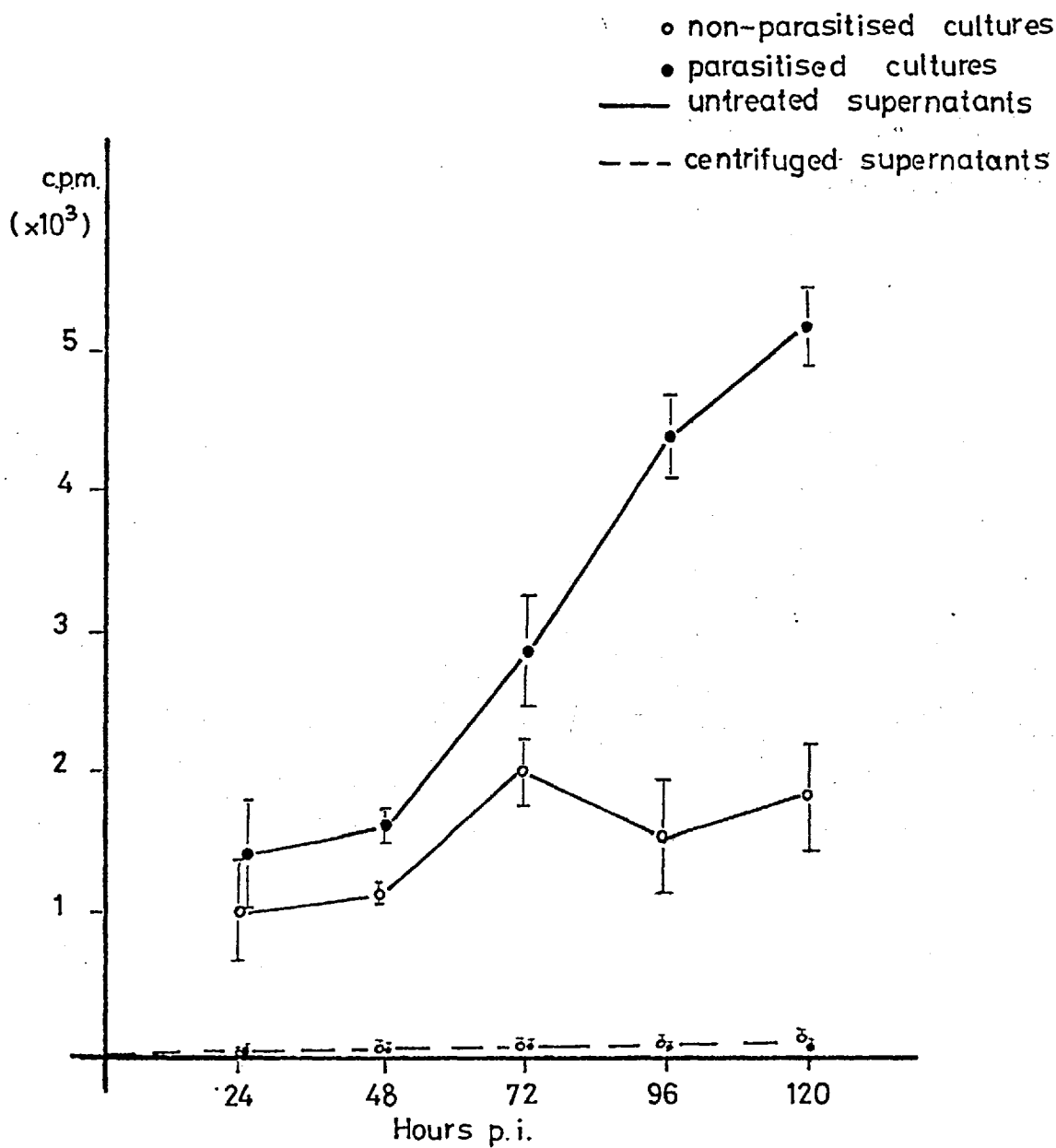
SECTION 4: DISCUSSION

4.1 Tissue Culture

4.1.1 Development of E. tenella in non-host cells from a sporozoite

inoculum

Development of E. tenella in non-host cells was poor with no

development occurring after invasion (Table 3.1.2), and in some cell

lines no intracellular sporozoites were detected. Mammalian cell

lines were routinely cultured at 37°C and were unable to adapt to

the optimal temperature for E. tenella development (41°C). All

cell lines except MDBK grew badly at 41°C and many cells were

detached after 48 hours at this temperature in experimental and

control cultures. These conditions probably account for the

failure of the parasite to develop in Flow 11,000, McCoy, MDCK and

bladder epithelial cells. The loss of cells in these cultures could

have obscured actual sporozoite invasion and is a possible

explanation for the lack of intracellular sporozoite at 24 hours p.i.

in some cell lines.

Previous attempts to grow E. tenella in mammalian cell lines

have met with limited success. Matsuoka et al (1969) used EBTr cells

grown at 41°C and reported that the cells remained in good condition

and supported the growth of mature second generation schizonts. The,

authors mentioned that cell lines in the 35-45th passages were best

for parasite development and that after the 50th passage the cells

began to deteriorate and cell proliferation slowed down. In the

present study, cells from the 55th passage and after were used, which

could explain why they were 1) less adaptable to temperature change

and 2) less able to support E. tenella development.

Shibalova (1970) reported development of mature first

generation schizonts in HeLa cells. No mention was made in her

report of the temperature at which the cells were grown. However,

this cell line failed to grow at 41°C in my experiments and although

intracellular sporozoites were observed, no further development

took place.

Patton (1965) found that MDBK cells grown at 41°C supported

development of mature first generation schizonts of E. tenella. In

the present investigation sporozoites, which had penetrated the

cells failed to develop further although the cells themselves

remained in good condition. It has been suggested that in CK

cultures E. tenella prefers to develop in islets of epithelial cells

and that such areas are necessary for gametogony, (Bedrnik 1967b;

Doran 1970a,1971c, Strout and Ouellette, 1970). Doran (1971c)

later suggested that it was not the epithelial nature of these

islets that was the important factor but rather the depth of tissue

which they could provide. This concept is supported in the present

work where parasites, particularly of the second generation, showed

a preference for thick layers of cells (Fig. 3.1.19). The fact that

MDBK and other cell lines are "trained" to grow as contact-inhibited

monolayers may explain the failure of these cells to provide a

satisfactory site for development of E. tenella. It would be

interesting to see if the growth of the parasite can take place in

the transformed counterparts of these cell lines as these exhibit

overgrowth of cells.

The inability of most cell lines to adapt to an increase in

temperature, together with their monolayer type of growth, suggests

that they are not a satisfactory means of growing and studying E. tenella

in vitro.

4.1.2 Development of E. tenella in host cells from a sporozoite inoculum

The greatest development of E. tenella was achieved in kidney cells, derived from either adult or embryonic tissue. Embryonic liver supported development to the mature second generation schizonts, but seemed to lack the necessary requirements for gametogony.

Development in cells derived from other types of embryonic tissue met with varied success though no stages past mature first generation schizonts were observed. No reports of the use of embryonic intestine, lung and spleen for the culture of E. tenella have previously been published, although mature first generation schizonts of E. meleagrimitis were obtained in turkey embryonic intestinal cells, (Doran and Vetterling, 1968).

Oocysts were found in only one culture of CEK cells, and were derived from a drug-resistant strain (Fig. 3.1.33). In previous reports of oocyst production in CEK or CK cells the macrogametes and microgametocytes have always been described as growing in profusion and very close together. The degree of intimacy varied with reports. Thus Doran (1970a) described them as growing very close together, Klimes et al (1972) described multiple nests of gametocytes and Itagaki et al (1974) said they were often to be found in the same parasito-phorous vacuole. Fertilisation occurred frequently in these clusters, to produce oocysts (Itagaki et al 1974).

The general failure of oocysts to develop in the present study could be attributed to one or more reasons. The cell layer had often deteriorated badly by the seventh day. This in turn would lessen the chance of surviving gametocytes growing to maturity and the accompanying

loss of cells, many of them parasitised, would further reduce the numbers of gametocytes present. Why the macrogametes should have failed to reach maturity when mature microgametocytes were often observed is difficult to explain. Gametocytes were rarely found growing close together and free microgametes were not seen. The isolation of macro- and micro-gametocytes may have diminished the possibility of fertilization of gametes.

It seems likely, therefore, that the failure to produce oocysts in cultures containing gametocytes was due to a combination of loss of parasitised cells, lack of maturity of the macrogametes and the absence of a close proximity of developing gametocytes.

The appearance of small schizonts at the time of gametogony, the intermediate size of their merozoites and the fact that they were only seen at days 6 and 7 p.i. indicated that they were of the third generation. Although two asexual generations is normal, Tyzzer (1929) described a third generation in vivo and Doran (1970a) and Strout and Ouellette (1970) described the appearance of third generation schizonts in vitro.

4.1.3 Growth in irradiated CK cells

Growth of E. tenella was limited in irradiated CK cells. These cells had their DNA synthetic mechanism damaged by irradiation as shown by their failure to take up ^{3}H-thymidine (^{3}H-TdR). Induction of an enhanced uptake of ^{3}H-TdR by the host cell nucleus in parasitised cells has been demonstrated in vivo for E. tenella (Fernando et al, 1974; Beyer and Shibalova, 1974) and E. necatrix (Fernando et al, 1974) and in vitro for E. tenella growing in MDBK cells (Browning et al, 1976) and in CEL cells (Latter, 1977). Furthermore, results in Section 3.3.3 indicated that a developing parasite induces and maintains the

high level of incorporation of ^{3}H-TdR by the host cell nucleus

during its intracellular development. A developing parasite must

necessarily place a big demand on the host cell for nutrition and

supply of metabolites and so any host cell which cannot respond by

increasing its protein output as a direct result of increased trans-

cription of RNA, will fail to support the parasite's development.

This increased RNA transcription would be facilitated by the production

of more than one copy of DNA, as signified by the enhanced uptake of

^{3}H-TdR. Irradiated cells would not be able to respond in this way,

and the failure of the parasite to develop in these cultures suggests

that such a response is a necessary requirement placed upon the host

cell by the parasite.

4.1.4 Growth of drug-resistant strains of E. tenella in vitro

Little difference was seen in the growth of normal and 'Deqox'

(decoquinate) resistant strains after 7 days in culture. However,

using the same strains, oocyst production from birds was slightly

lower in the normal strain. Augustine, Vetterling and Doran (1977)

found growth in culture and oocyst production in chickens were

similar when they compared a normal with a buquinolate-resistant

strain of E. tenella. Buquinolate and decoquinate are similar

compounds both being anticoccidial quinolones.

The Pancoxin resistant strain grew better in culture than the

normal strain of E. tenella at 7 days p.i. and also gave a higher

output of oocysts from chickens. Pancoxin contains 18% amprolium,

10.8% sulphaquinoxaline and 0.9% ethopabate. All three drugs inter-

fere with cofactor synthesis, amprolium affecting thiamine pyro-

phosphate production by inhibiting the uptake of thiamine by the

parasite (Ryley 1972) and sulphaquinoxaline and ethopabate affecting

the synthesis of tetrahydrofolate (Gutteridge and Coombs, 1977).

In respect of growth in culture and oocyst production in chickens Augustine et al (1977) found no differences between a normal strain of E. tenella and one which was resistant to amprolium. They found that initially amprolium-resistant strains showed a delayed development of the first generation schizonts, despite a similarity of invasion by the sporozoites at 24 hours p.i. This difference was no longer evident at 72 hours p.i. Augustine et al (1977) attributed the delayed schizogony to biochemical alterations in the resistant strains. Such alteration they postulated made the strain less dependent upon thiamine by either i) allowing it to function within existing pathways at lower than optimal levels or ii) causing it to develop alternative but less efficient, metabolic pathways to replace those that required thiamine. Thiamine is known to be necessary for gametogony (Warren, 1968) as well as schizogony (Strout and Ouellette, 1973). It is, therefore difficult to see how retardation at the schizogony stage would not have been repeated at gametogony, if as they suggested, the parasite was developing at a lower level of metabolism.

As Pancoxin is only 18% amprolium, and contains two other anti-coccidial drugs (sulphaquinoxaline, 10.8%. and ethopabate, 0.9%) the development of the Pancoxin-resistant strain in chicks and in cell culture is not strictly comparable with development described for the amprolium-resistant strain by Augustine et al (1977). Furthermore, Augustine et al (1977) used the strain E. tenella (Beltsville), whereas in this study E. tenella (Weybridge) was used. It is conceivable that selection from E. tenella (Beltsville) for strains resistant to amprolium alone, produced a mutant which differed from the mutant

selected from E. tenella (Weybridge) for resistance to amprolium, sulphaquinoxaline and ethopabate.

Alternatively, differences could have been due to the type of medium used to maintain the cells during parasite development. Augustine et al (1977) used Hank's Balanced Salt Solution (HBSS) with 5% lactalbumin hydrolysate (L.H., 2.5% solution in HBSS) and 5% foetal calf serum. In the present study the maintenance medium was Medium 199 (M199) with 1% Yeast (4% solution in HBSS) and 1% calf serum. M199 is a much more nutritious medium and could have encouraged the exploitation of the alternative pathways. However, as medium differences cannot account for the differences in oocyst production in chickens in the two reports, it is more likely that differences existing between these two amprolium-resistant strains were due to their selection from two different parent strains.

4.1.5   Morphology of schizonts and gamonts of E. tenella

The morphology of the schizonts observed in this study corresponded to the two basic patterns described for E. tenella by Strout and Ouellette (1970). The conventional schizont, resembling the in vivo type, had the merozoites arranged in a rosette pattern around a residual mass. The haphazard arrangement often seen in schizonts (Figs. 3.1.17 and 3.1.23) probably represented a later stage of this type of schizont development. Kheysin (1972) suggested that the haphazard arrangement of merozoites was due to movement around the residuum. Furthermore as merozoites are known to become spontaneously activated once they have broken away from the residuum (Strout and Ouellette, 1970) the haphazard arrangement is probably indicative of merozoites just prior to their release.

The second type of schizont was the blastophore type described in vitro for E. tenella by Itagaki et al, (1974); Long, (1969); Strout and Ouellette, (1970). It is not known to occur in vivo for any avian coccidia, although it is characteristic of the in vivo development of E. bovis from cattle, (Hammond, Ernst and Miner, 1966) where schizonts reach large sizes (300 x 200 μm). Blastophore formation may be a mechanism for ensuring that nutrients more easily reach all parts of the schizont, or may provide a greater surface area for budding of merozoites. Itagaki et al (1974) stated that ... "the blastophore may be an unusual form which has developed in the abnormal environment of cultured cells, ... and may be concerned with the intake of nutrients"... Apart from this explanation no other reasons for their development in cell culture have been proposed. They are unlikely to develop as a mechanism for coping with size as the dimensions of schizonts in vitro are not markedly different from those in vivo. The blastophore schizonts of E. tenella are not strictly comparable to those of E. bovis. In E.bovis the nuclei become dis-tributed in a single peripheral layer and the surface of the schizont becomes infolded to form compartments. These themselves acquire a peripheral layer of nuclei (Hammond, 1973). In E. tenella blastophores the nuclei are found distributed throughout the cytoplasm and are not limited to the surface layer, suggesting they developed from a disin-tegration of the schizont body into smaller bodies and did not result from infolding of peripheral nuclei.

Microgametocytes were also seen in a subdivided form. Although this morphology has not been mentioned in vitro before, Kheysin (1972) described their appearance in vivo and called them blastophores,

equating them with the microgametoblasts of Tyzzer (1929). In this
case they could be expressing a response by the microgametocyte to an
abnormally large size, as the microgametocytes often reached much
larger sizes in vitro than in vivo (Tables 1.4 and 3.1.5.).

4.1.6 Sizes of intracellular stages

Previous workers have reported the first generation schizonts
and merozoites in vitro to be larger than in vivo (see Table 1.4).
In the present study the schizonts appeared larger than in vivo
(44 x 22µm compared with 24.0 x 17.3µm) and the merozoites were com-
parable in size, or slightly larger.

The second generation schizonts were comparable in size with
those found in chicks, when they developed in isolation. Quite
commonly there was invasion of limited areas by a profusion of
schizonts and these were much smaller than usual. The merozoites of
all types of schizont were smaller than in vivo which accords with
results from other work (Table 1.4).

The macrogametes were comparable in size to those appearing
in vivo but microgametocytes grew much larger. The sizes of the
microgametes could not be measured accurately as they were never to be
found lying clear of the general mass.

The sizes of the third generation schizonts were slightly larger
(19.7 x 18.6µm) than those described previously in vivo. (9 x 7.6µm;
Pellérdy, 1974).

4.1.7 Development of E. necatrix in CK cells from a sporozoite inoculum

No development occurred although previous reports have shown growth
to mature second generation schizonts (Doran 1971a) in CK cultures, and
the production of oocysts in CAM tissue (Shibalova, 1970). The failure

here is not easily accountable for, especially as a considerable number

of sporozoites entered the cells. The oocysts were only a month old

and thus deterioration of sporozoite viability should not have been a

problem.

4.1.8 Development of E. necatrix from second generation merozoites obtained

from the chick

Merozoites of E. necatrix failed to enter CK cells and mouse

macrophages. Mouse macrophages were chosen as previous, unpublished,

results (Sinden, personal communication) indicated massive invasion,

by E. tenella sporozoites, into these cells over 24 hours. Because of

this observation the failure of all merozoites to enter, or be taken

up, by macrophages is surprising. They were carefully prepared

following the method of Stotish and Wang (1975) and were motile when

applied to the cultures. Merozoites are known to be more sensitive

than sporozoites. Bedrnik (1969b) found that they entered cells less

readily than sporozoites. Long and Speer (1977) also reported that

only a limited number invaded cells despite gentle handling and the use

of low concentrations of bile salts to stimulate activity. Failure of

E. necatrix merozoites to enter CK cells and mouse macrophages must

probably be explained on the basis of loss of viability of these

merozoites.

The extraction procedure to obtain the merozoites from chickens

was only performed a few times and previous experience with the

preparation of sporozoite inocula, indicated that the development of

sporozoites in vitro improved with practice. Therefore it would be

wrong to assume that E. necatrix merozoites were incapable of invading

and developing in the cells until the test has been repeated a few

more times.

4.2 Invasion into Cells

4.2.1 Invasion into synchronised cells

A synchronised population of cells, with most of the cells in, or about to enter, S phase was more heavily infected with sporozoites of E. tenella, than was an asynchronous population. This suggested that sporozoites have a predilection for cells in the S phase or for those at the G_1/S boundary. A method by which this could be confirmed would be to conduct labelling experiments with ^3H-thymidine (^3H-TdR) in conjunction with invasion studies: a higher percentage of infected cells containing labelled nuclei compared with non-infected cells would indicate the preference of sporozoites for cells in S phase.

Sporozoites are known to enter and leave cells rapidly in culture before they finally remain intracellular and begin development (Doran 1973). Long and Speer (1977) suggested this was also a feature of in vivo development, with E. tenella sporozoites entering and leaving cells on their journey down the intestine, settling down only in the epithelial cells of the caeca. It could be that sporozoites are able to recognise an environment suitable for their development once they are intracellular and leave the cell if their needs are not met. The idea that they only respond to their environment after entry suggests that they cannot recognise cells in the preferred stage of the cell cycle by their cell surface properties.

The preference for G_1/S and S phase cells shown by the invading sporozoites in culture may be related to the type of cell they prefer in vivo. E. tenella sporozoites are known to enter and develop in epithelial cells with a proliferative capacity, (Fernando et al 1974). These cells are found at the base of the intestinal crypts. Epithelial cells are eventually sloughed off and lost as they move up the side of the crypt towards the tip of the villus. By choosing these cells

E. tenella can complete the first generation schizogony before the

host cell is sloughed off.

Proliferative cells have the capacity to enter S phase and

E. tenella stimulates an uptake of ^{3}H-TdR by the host cell nucleus

(Browning et al 1976). Results from the present study (see Sections

3.1.4 and 3.3.3) indicate that this response is a prerequisite for

further development of the sporozoite. It is possible that sporo-

zoites can recognise a factor in the cytoplasm of S phase cells, or

those near the G_1/S phase boundary, which indicates that the cells

have the capacity to take up ^{3}H-TdR. There is evidence from other

cell systems that the trigger to start DNA synthesis is located in

the cytoplasm of a cell (Mitchison, 1973).

4.2.2. Mechanism of Invasion into cells

New born calf serum and donor horse serum lowered the invasion

of sporozoites into cells; chicken serum was not inhibitory. With

the heterologous types (i.e. new born calf and donor horse) the degree

of inhibition increased with the concentration of serum added to the

inoculation medium. This inhibition was substantially reduced when

the serum was inactivated by acid or heat-treatment. Chicken serum

was less inhibitory to sporozoite invasion than the heterologous sera

regardless of whether it was inactivated or not. Serum is thought to

affect the viability of sporozoites (Browning et al 1976) and the

lowered invasion observed in the presence of serum could have been due

to their loss of viability. However, the results from the pre-incubation

of sporozoites with serum showed that the fall off in invasion of cells

by sporozoites could not be attributed entirely to their loss of

viability.

It is possible that the protease inhibitors in heterologous normal

serum inactivate proteases produced and used by sporozoites during the invasion process, and that these proteases remained unaffected by protease inhibitors present in chicken serum. The involvement of protease inhibitors was tested by using an exogenous supply of inhibitors, added to the inoculation medium in the absence of serum. All the protease inhibitors tested lowered the invasion by E. tenella sporozoites to a greater or lesser extent (Figs. 3.2.7 to 3.2.11) and usually in a dose-dependent manner. Pre-incubation of sporozoites with the protease inhibitors showed that these inhibitors were not causing any significant loss of sporozoite viability.

Sporozoa possess an apical complex located at the anterior end of the motile stages (Scholtyseck et al, 1970; Scholtyseck and Mehlhorn, 1970). The secretions of this apical complex (which includes rhoptries and micronemes) have been implicated in the invasion process (see Section 1.3). Possibly the lowered invasion observed in this study was due to a direct action of the protease inhibitors on the rhoptry-microneme secretions. The nature of these secretion remains equivocal. The original suggestion that they are proteolytic substances (Garnham et al 1960; Scholtyseck and Mehlhorn, 1970) could explain the present results.

Jensen and Edgar (1976) suggested that the secretions from the rhoptries of E. magna may resemble similar rhoptry products believed to be associated with Plasmodium invasion. A histidine-rich polypeptide has been isolated from P. lophurae merozoites (Kilejian, 1976). This protein induced changes to the membranes of duck erythrocytes consistent with a possible role of inducing invagination of the erythrocyte membrane. Furthermore, Jensen and Edgar (1976) drew attention to the similarity between alterations to the parasitophorous vacuolar membrane

which they observed in ultrastructural preparations of E. magna and changes induced by polycationic polypeptides in erythrocyte membranes (Shotton et al, 1975). Polypeptides, with similar characteristics to those from P. lophurae have recently been demonstrated in T. gondii (de Souza and Souto-Padrón, 1978).

Bannister et al (1975) proposed that rhoptry and microneme products of P. knowlesi merozoites were incorporated into the erythrocyte membrane and by disordering the phospholipid bilayer produced the inward invagination associated with merozoite invasion. This hypothesis has since been elaborated (Bannister et al 1977) with the suggestion that these products are preformed lamellae which are incorporated directly into the host cell membrane. Results from freeze fracture studies have indicated that this material is lipid-rich/protein-poor (McLaren et al, 1979). Jensen and Edgar (1976) suggested that as an alternative to being polypeptides the secretory products of E. magna rhoptries may resemble those of P. knowlesi (Bannister et al 1975).

In view of the mounting evidence against proteases being involved directly in invasion, the inactivation of protease inhibitors described here must be considered from a different viewpoint.

If the rhoptries of Eimeria spp. contain a polycationic polypeptide similar to that described for P. lophurae (Kilejian 1976) and T. gondii (de Souza and Souto-Padrón 1978) it is likely to be produced and stored in an inactive form. If it were present in the active form whilst still in the rhoptries and micronemes, such a heavy concentration of the polypeptides would be likely to cause total disorganisation of the membranes surrounding these organelles. Enzymes may therefore be present in the rhoptries and micronemes to function as activators for

the stored polypeptides. The exogenously supplied protease

inhibitors, and those present in the untreated serum, may have

prevented invasion by inactivating these enzymes.

Alternatively if the contents of the rhoptries are formed into

lamellar structures prior to invasion, protease inhibitors could be

inactivating enzymes involved in this process.

The nature of the rhoptry and microneme products of E. tenella

sporozoites must remain equivocal until biochemical analyses are

performed on purified contents. However, the results obtained here

are not inconsistent with the idea that this product is stored in an

inactive form, which can be activated by proteolytic enzymes also

found in the rhoptries.

4.3 Host Cell Changes

4.3.1 Morphological and Adhesive Changes

Changes to the morphology and the general appearance of cells from

parasitised cultures were evident 1 day post-inoculation. They became

gradually more widespread with time and appeared to affect parasitised

and non-parasitised cells alike. The general deterioration in the

condition of cells when parasitised has been described previously

(Bedrnik 1969a; Doran and Vetterling 1967b, 1968; Itagaki et al 1974).

Clark and Hammond (1969) mentioned that, although cytopathological

changes were initially limited to cells parasitised by E. auburnensis,

they gradually spread to affect the entire monolayer. They suggested

that a toxic substance produced by the parasite was responsible for

this change.

By using the scanning electron microscope the gross morphological

changes were observed in detail. Firstly the cells began to lose

contact with one another, remaining attached initially by thin cyto-plasmic bridges but eventually breaking away completely. Following this they began to round up, appeared to "lift" away from the substrate, and eventually remained attached to the substrate only by means of long retraction fibres. The ends of the retraction fibres represented the original points of adhesion of the cells to the substrate, (Revel, Hoch and Ho, 1974). Eventually the cells became completely detached as indicated by the large areas of the coverslips devoid of cells and the presence in these areas of cellular débris, representing the remains of the attachment sites (Badley, Lloyd, Woods, Carruthers, Allcock and Rees 1978).

Reflection interference microscopy (RIM) was developed as a technique for analysing the separation distance of cells from their substrates (Curtis 1964). Studies by RIM confirmed that there was an increase in the distance between the cell and its substrate in those cells affected by the parasites. All three basic types of contact between cells and their substrates, described by Izzard and Lochner (1976),were seen in the CK cells grown in this study, but the pattern and distribution varied considerably between parasitised and non-parasitised cultures. In non-parasitised cultures, focal contacts, indicating distances of 10-15 nm and represented by dark streaks, were arranged in a neatly aligned manner at the edges of the cells, or the cell sheets. They occurred less frequently at the interior of the cells. The central regions were characterised by areas producing grey images (close contacts) which represented a separation distance of about 30nm. Interspersed with these were occasional areas producing a white image; here the cell was lying about 100-140 nm away from the substrate.

In parasitised cultures this pattern was virtually destroyed and replaced by a disorganised array of images. Focal contacts were rare; rather there was a predominance of dark grey-grey areas, and an increase in the area of the cells producing white images. These facts together with a notable rise in the regions which produced no image at all (and were therefore lying more than 140 nm away from the substrate) showed that parasitised cultures contained cells which were less well adhered. These RIM observations supported those obtained by SEM.

Of several factors involved in cell adhesion (Curtis 1964; Edwards, 1977) one is the long range physical forces. These may be involved in drawing cell-cell and cell-substrate approaches together to allow the formation of chemical bonds to occur. The focal contacts represent the sites of formation of chemical bonds. By lowering the electrolyte concentration of the medium the long range physical forces should extend further from the cell, with the result that the separation from the substrate would increase and adhesion decrease (King, Heaysman and Preston 1979). When the CK cultures were perfused with 0.25M sucrose to lower the electrolyte concentration the RIM images showed that the cells lifted from the substrate. Parasitised and non-parasitised cultures responded initially in a similar way. However, with continued perfusion a difference became noticeable. In the non-parasitised cultures the focal contacts remained and became even more clearly defined against the surrounding grey and white areas. This effect was reversible, with the RIM image reverting to normal when the sucrose was replaced with M199. With parasitised cultures the original dark grey-grey areas around the edges of the cells and cell sheets were lost with

continued perfusion with 0.25M sucrose and only a few focal contacts remained. Some cells lifted almost clear of the substrate eventually producing no image at all (Fig. 3.3.23). These changes were not reversible in most parasitised cultures, indicating that these cells had lost their chemical adhesions and could therefore respond to the alterations in electrolyte concentration in a less restrictive manner than the cells from non-parasitised cultures.

From both RIM and SEM studies it was apparent that the morphological and adhesive alterations affected all cells in a parasitised culture regardless of whether they contained a parasite or not. The morphological changes observed by SEM could also be seen in non-parasitised cells grown in coculture with parasitised cells and must therefore have resulted from factors transmitted in the medium from parasitised cells. The factors could either be products of an intracellular parasite or of a parasitised cell. The nature of these products is undetermined but a possible site of their action can be suggested. The shape of a cell is generally held to be a feature of its cytoskeleton. This consists of three main components, the actin-containing microfilaments, 10 nm filaments, and microtubules. This assembly lies just under the cell membrane and by interacting with it through a variety of intermediary contacts it is believed to control many aspects of a cell's shape (Loor 1976). The spreading and flattening of a cell seems to be a property of its microfilament system with the microtubules playing a supportive role in the maintenance and development of cell shape but not actually involved directly in the spread or adhesions of a cell (Lloyd, Smith, Wood and Rees 1977). Disorganisation of actin filaments has been correlated

with the move from a flattened to a rounded morphology (Damskey,
Wylie and Buck 1979; Malluci and Wells 1976; Pastan and Willingham
1978; Willingham, Yamada, Yamada, Pouysségur and Pastan 1977). It is
therefore conceivable that the change from a flattened, well spread
shape to a more rounded appearance seen in the parasitised and
cocultured non-parasitised cells was due to disorganisation of the
actin filament bundles. Further, adhesion is closely linked with
cell shape because bundles of microfilaments terminate at the edges
of cells in the focal contacts, (Abercrombie and Dunn, 1975; Izzard
and Lochner, 1976; Heath and Dunn, 1978; Wehland, Osborn and Weber, 1979).
The grey areas or close contacts are regions of less organised
microfilaments (Heath and Dunn 1978). Therefore the change in RIM
images seen when cells were parasitised is consistent with the idea
that the microfilament bundles had become disorganised due to some
factor, a product of parasitised cultures, present in the overlying
medium.

A possible explanation of the apparent anomaly between the
expression of morphological changes but not the adhesive changes by
cocultured non-parasitised cells, even though both alterations are
attributable to microfilament disorganisation, lies in the theory of
cell adhesion proposed by Rees et al (1977). They proposed that
adhesion was determined by two factors, (a) the internal control
('grip') exerted by the cytoskeleton and (b) the external control
('stick'). The grip control they further subdivided into a bracing
grip, which was responsible for the overall shape and stiffness of
the cell and a focal grip, which held the molecules involved in cell-
substrate adhesion in an organised configuration at the site of contact
of the cell with its substrate. They believed that bracing and focal

grips were capable of acting independently of each other. It is possible, therefore that in cocultured non-parasitised cells the bracing grip had been altered, to give the rounded shape, whereas the focal grip remained untouched, hence the retention of the focal contacts, as seen in the RIM of cocultured non-parasitised cells.

External glycoproteins, thought to be involved with cell-cell and cell-substrate adhesions, are intimately associated with the microfilament bundles in the focal contacts (Lloyd, 1979, review). Recently a glycoprotein fibronectin, has been demonstrated to be linked with the microfilaments inside the cell (Hynes and Destree 1978) and breakdown of microfilament bundles by means of cytochalasin B, caused release of fibronectin from the cell surface (Kurkinen, Wartiovaara, and Vaheri 1978). Fibronectin is known to be involved in the formation and maintenance of cell adhesions (Yamada and Olden 1978) and to induce cell spread (Willingham et al 1977; Ali, Mautner, Lanza and Hynes, 1977). In the parasitised cultures loss of glyco-proteins caused by disorganisation of microfilaments could be responsible for a fall in adhesion and detachment of cells.

In summary, it is proposed that an initial depolymerisation of microfilament bundles may cause a loss in cell-cell and cell-substrate adhesions, allowing the cells to round up. With further disorganisation at the actual sites of contact of the cell with its substrate (the 'feet' of Revel et al 1974), the chemical 'glue' may be dissolved, with the result that the cell detaches and floats away.

4.3.2   Enhanced agglutinability with ConA

Decrease in adhesion and cell spread in other cell systems has often been accompanied by increased agglutination of cells with plant

lectins (see review by Nicolson, 1974). The CK cultures parasitised

by E. tenella show an increase in the degree of agglutination with

ConA, a lectin which is isolated from the jack bean (Nicolson, 1976).

The agglutination of CK cells was sometimes difficult to assess

due to a fairly high level of background adhesion apparent in the

control groups. The inclusion of DNAase in these controls eliminated

the possibility that the clumping resulted from leakage of DNA during

the preparation of the cell suspension (Schnebli 1976). Adhesion,

although a separate phenomenon from agglutination, may influence

agglutination by allowing cells to move near enough to facilitate the

formation of agglutinin bridges (Walther 1976). Furthermore, epithelial

cells are known to show a certain degree of spontaneous aggregation

(Schnebli 1976). These two factors could explain the slight agglut-

ination seen in non-parasitised cells.

Increases in agglutinability with plant lectins has been attributed

to the ability of their cell-surface receptors to cluster in the cell

membrane (Guerin et al 1974) with clustering being facilitated by an

increase in membrane fluidity (Rosenblith, Ukena, Yin, Berlin and

Karnovsky 1973). Experiments where cells were prefixed with glutar-

aldehyde before agglutination seemed to confirm this idea. Inbar et al

(1976) suggested that, as prefixation did not lower the amount of ConA

bound to cells, but did lower agglutination, then the ability of the

receptors to move from a random distribution to a clustered one was

necessary for agglutination to occur. In the present study, pre-

fixation of cells from parasitised cultures did lower the agglutin-

ability with ConA so a similar change in receptor mobility in the

membrane could be implicated here. However, as no experiments were

performed to see if less ConA was bound to fixed cells, the fact that reduced agglutinability was due to a reduced amount of bound ConA cannot be excluded.

Recently the control of membrane fluidity on receptor mobility has been questioned (Hatten, Scandella, Horwitz and Burger, 1978). Rather a transmembrane control of cell surface receptor mobility would appear to be of prime importance (Clark and Albertini, 1976; Edelman, Wang and Yahara, 1976; Nicolson, 1976). Edelman et al (1976) described this control as a function of a surface modulating assembly, (SMA). The SMA they said was tripartite, consisting of certain surface receptors, microfilaments and associated myosin, and microtubules. It served as a mediator of cell surface receptor-cytoplasmic interactions, and any alterations in the association of the microfilaments and micro- tubules with the cell membrane would be likely to involve a redistri- bution of cell surface receptors. Furthermore, ConA-binding sites have been shown to be associated with microfilaments in ovarian granulosa cells (Clark and Albertini, 1976).

With these reports in mind it is possible that the disorganisation of microfilaments, suggested as the cause of the changes in morphology and adhesion in parasitised cultures, could also be the cause of increased agglutinability with ConA seen in these cells.

4.3.3 Release of glycoproteins

CK cells, pre-labelled with ^{3}H-1-fucose, were inoculated with E. tenella sporozoites and the changes in associated radioactivity compared. over 5 days p.i., with non-parasitised cultures. Fucose is a monosaccharide sugar and is mainly incorporated into the cell surface glycoproteins in a terminal position (Watkins et al 1962). Virtually

none is metabolised into other glycolytic products, neither is it

used in alternative biosynthetic pathways (Bekesi and Winzler, 1967;

Coffey, Miller and Sellinger, 1964). When HeLa cells were labelled

with ^{3}H-1-fucose 70-100% of the total T.C.A. insoluble fraction was

associated with the plasma membrane (Atkinson and Summers, 1971).

Furthermore, Buck et al (1970) demonstrated that 90% of the fucose

label incorporated into BHK cells was recovered as labelled fucose.

In view of these results, it was assumed in the present study that

most of the ^{3}H label was still associated with fucose.

^{3}H-1-fucose was rapidly lost from parasitised and non-parasitised

cells over the first 24 hours, with only a slight fall occurring in

both these cultures after this time. An increase in radioactivity

associated with the T.C.A. precipitable fraction of the supernatants

from both these cultures occurred, and became more pronounced in the

parasitised cultures with time. In a previous report (Urquhart, 1979)

this higher level of radioactivity in the supernatant of parasitised

cultures was attributed to a greater release of glycoproteins from

the cell surface, in response to a proteolytic activity in the medium.

This conclusion is now open to some doubt in the light of present

findings, when the differences between the radioactivity associated

with the supernatants was eliminated by spinning down the supernatants

before T.C.A. precipitation. This indicated that the greater radio-

activity in the supernatants from parasitised cultures could be almost

entirely attributed to the greater loss of whole cells, and membrane

fragments as a result of adhesive changes, rather than to glycopeptide

segments being released from the cell surface glycoproteins as a

result of proteolytic activity.

4.3.4   Enhanced uptake of ^{3}H-thymidine

Parasites stimulated an uptake of ^{3}H-thymidine (^{3}H-TdR) in their

host cell nuclei. This was evident by 16 hours p.i. Parasites them-

selves remained unlabelled throughout the 72 hours of the experiment.

This agreed with previous reports that Eimeria spp. were unable to

utilise exogenously supplied thymidine (Roberts, Elsner, Shigematsu

and Hammond, 1970; Ouellette, Strout and McDougald 1973, 1974;

Morgan and Canning 1974). A stimulation of the host cell nuclei

following parasitisation has previously been described in vivo

(Fernando et al 1974; Fernando and Pasternak 1977; Beyer and Shibalova

1974) and in vitro (Browning et al 1976; Latter, 1977).

The increase in DNA synthesis, although associated with nuclear

hypertrophy, is unaccompanied by cell division (Beyer and Shibalova

1974; Fernando et al 1974). The increase in DNA synthesis is likely

to be a response of the host cell to cope with the extra demands placed

upon it by the parasite.

Previous reports of in vitro stimulation of ^{3}H-TdR uptake were

those of Browning et al (1976) who described the effect of E. tenella

on incorporation of ^{3}H-TdR by MDBK cells, and of Latter (1977) who

described the effect of developing and differentiating second

generation schizonts of E. tenella on ^{3}H-TdR incorporation by CEL

cells. Browning et al (loc. cit.) mentioned that all cells in a

parasitised culture were stimulated to take up thymidine regardless

of whether they were parasitised or not, and attributed this to a

diffusible substance. This was confirmed in the present work by

showing that the stimulus for enhanced ^{3}H-TdR uptake could be trans-

mitted via the medium to non-parasitised cells in coculture. These

non-parasitised cells responded in exactly the same manner as the

parasitised cultures except that the level of incorporation was not

quite as high. Parasitised cultures showed enhanced uptake at 16 hours p.i. (4 hours of coculture), when they were free of invading sporozoites. There was no time lag in response of cocultured non-parasitised cells to the stimulus as they showed enhanced uptake within 4 hours of coculture.

Further evidence for a diffusible factor causing this stimulation comes from a comparison of the labelling indices of non-parasitised cells from parasitised cultures (mixed cultures) with the labelling indices of the non-parasitised cells in coculture. The labelling indices were identical, suggesting that cell-cell stimulation was not as important as cell-medium-cell stimulation. In vivo, this extra-cellular, diffusible factor, which enhances thymidine uptake, is unlikely to affect neighbouring non-parasitised cells as it would be diluted, and possibly inactivated, by gut fluids and eventually removed from the area by the general movement of the gut contents. Fernando et al (1974) did in fact mention that in vivo there was no evidence to suggest that uninfected cells lying adjacent to schizont-infected cells received a signal to initiate schizogony. However, they believed that the induction of DNA synthesis in the host-cell nucleus was due to the presence of a short-lived factor retained in the host cell at low concentrations.

In vitro and in vivo DNA synthesis by parasitised cells has undoubtedly been placed under a new set of controls. The coculture results obtained here have provided some evidence for the course of events leading to this induced and prolonged DNA synthesis. Over the first 24 hours the ratio of labelled to non-labelled nuclei was identical for i) parasitised and non-parasitised cells in the mixed

cultures (Fig. 3.3.31) and ii) non-parasitised cells from mixed and cocultures (Fig. 3.3.30). After 24 hours the parasitised cells showed an increased proportion of labelled nuclei whereas the proportion fell for both types of non-parasitised cells. Therefore, although initial stimulation of uptake of ^{3}H-TdR was not dependent on the presence of a parasite it did depend on a transmissable factor in the medium. Maintenance of high levels of ^{3}H-TdR incorporation were dependent on the presence of a parasite, and the factor concerned with this maintenance could not transmit its effects via the medium.

Browning et al (1976) described little difference in the labelling indices of parasitised and non-parasitised MDBK cells in their mixed cultures after 24 hours. Although this contrasts with the present results where there was a distinct difference, there is a possible explanation. Few developing stages were found in MDBK cells and, in the absence of developing parasites, the necessary factors which maintain high levels of ^{3}H-TdR incorporation would not be produced and the incorporation would fall in parasitised cells. The abundance of developing stages in the present CK cultures would be associated with the production of the secondary factors and this would in turn enable the parasitised cells to maintain a high level of incorporation.

In conclusion it would appear that parasites which alter the DNA synthetic processes of their host cells or parasitised cells produce two factors. The first is released into the medium and induces other cells to enter S phase and take up ^{3}H-TdR; its production ceases soon after invasion. Later a second factor is produced which is not released, but serves to retain a parasitised cell in S phase. It is obviously in the interest of the parasite to prevent its host cell undergoing

mitosis by retaining it in a pre-mitotic phase of the cell cycle.

4.3.5 Implications of the host-cell changes for the <u>in vivo</u> development of <u>E. tenella</u>

Changes of the cell surface and membrane observed in tissue culture must be regarded in the light of the role they may play in the life-cycle of <u>E. tenella</u> <u>in vivo</u>. The cells containing young second generation schizonts detach from adjacent epithelial cells and move into the subepithelial tissue of the villus. This detachment could be due to a loss of cell-cell adhesion, evidence for which is suggested in the tissue culture system. The migration of these infected cells also indicates an alteration in the specificity of adhesion because, although the attraction between epithelial cells must decrease, the epithelial cell-lamina propria cell attraction must increase. This may be expressed by an alteration to the cell surface properties.

The importance of the parasite's ability to induce, and maintain, extra rounds of DNA synthesis in the host cell nucleus has been discussed previously. Basically this ability is necessary i) to allow the host cell to cope with the increased demands upon its metabolism made by the developing parasite and ii) to ensure that the parasite's development will not be hindered or stopped by the host cell changing its metabolism in preparation for cell division.

4.3.6 The possible nature of the factor(s) which caused the alterations to the host cell's morphology, adhesion and cell cycle, and its implication in the study of cell regulation

A previous preliminary report suggested that a proteolytic substance produced by the parasite and released into the medium may have

been responsible for the host-cell changes (Urquhart, 1979). Despite

the now questionable role that proteolysis may be playing in the

^{3}H-1-fucose labelling studies, circumstantial evidence exists to

suggest that its involvement in some of these host-cell changes need

not be totally excluded. Proteolytic activity is known to i) induce

agglutinability with lectins in non-agglutinable cells (Burger 1969),

ii) breakdown the cytoskeletal structures of cells (Pollack and Rifkin

1975,1976), with a consequent loss of cell spread (Furcht and

Wendelschafer-Crabb 1978), iii) be associated with the release of

fibronectin/LETS protein from the cell surface (Hynes 1973) and iv)

induce DNA synthesis and cell division (Burger 1970, Sefton and Rubin

1970).

Further investigations could give some indications on the role

Ca^{2+} and/or cyclic nucleotides are playing in these host cell responses;

these two are generally held to have important roles in the regulation

of cellular responses. Therefore, this parasite-host cell in vitro

system is of great potential to cell biology, particularly in the

study of regulation and control of cellular activity.

4.4 General Conclusions

The present study has indicated that the growth of E. tenella

in vitro provides an ideal system for observing host-parasite

relationships both during invasion and development. By extending the

work on invasion antagonists, advances could possibly be made in

understanding the biochemical aspects of invasion. Present knowledge

of the effect of E. tenella on its host cell is very limited. The

results described here suggest that many of the host cells' fundamental

processes have been altered but there still remains a great deal to be

understood concerning this important topic. Only when basic facts

about the parasite's biology, and its interactions with the host,

are clearly understood, will the search for controls against this

important disease be able to follow a more directional and relevant

approach.

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225

Membrane changes in Chick kidney cells cultured
with Eimeria tenella.

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Chick kidney cells cultured in vitro undergo modifica-
tions to the cell membrane, when parasitised by Eimeria
tenella. Cells from parasitised cultures showed in-
creased agglutination with the lectin Con. A, 24 hours
after inoculation of the cultures with sporozoites.
This can be explained by increased lectin receptor
mobility within the membranes of cells in these cultures.
Mild proteolytic activity has been shown to induce
agglutination in cells normally insensitive to lectins,
and so the enhanced agglutination with Con A described
above could indicate the presence of proteolytic acti-
vity within the parasitised cultures.
Additional evidence for proteolytic activity was obtain-
ed from experiments which measured the release of glyco-
proteins labelled with L-3H-fucose from the cell surface.
A greater loss of radioactivity was detected from the
cells from parasitised cultures over a period from 48
hours to 5 days post-inoculation of sporozoites. Some
proteolytic enzymes have been shown to attack amino
acid residues in the peptide chains of cell surface
glycoproteins. This could explain the loss from the
cell surfaces described above. Membrane alterations
caused by E. tenella infections were visualised more
directly when the morphology of the cells was examined
with the scanning electron microscope. Cells from
parasitised cultures were less adherent to the cover-
glasses, allowing the cell boundaries to roll up and
produce a more angular outline than that exhibited by
cells from non-parasitised cultures. Many more cells
were affected than those actually containing parasites,
which indicated that the stimulus acted through medium.
Evidence that parasitised cells can release a factor
into the medium which can affect non-parasitised cells
was provided by experiments on thymidine uptake: the
uptake of 3H thymidine was enhanced in non-parasitised
cultures when they were grown in coculture with
parasitised cells.

THE CORRELATION BETWEEN PLASMINOGEN ACTIVATOR-STIMULATED DNA SYNTHESIS AND CELL MORPHOLOGY IN 3T3 CELLS

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SUMMARY

3T3 cells when grown in co-culture with SV40 3T3 cells under varying conditions of plasminogen activation displayed an increase in thymidine incorporation, suggesting growth stimulation, a decrease in area of cell spread, and loss of cell adhesion as measured by trypsin sensitivity. Evidence is presented that the mechanism whereby plasminogen activator initiates DNA synthesis is by enhancing serum stimulation; this is related to the concomitant changes in cell morphology which may allow greater access of serum to the cells.

The levels of plasminogen activator (a protease which converts serum plasminogen to plasmin [1]) have been shown to be higher in many transformed cells than in normal cells [2], and there is evidence that plasminogen activation is associated with the maintenance of several of the characteristic features of malignant cells. These include altered morphology and multilayering [3, 4], ability to grow in soft agar [3] and fibrinolytic activity [5], but evidence for an association between plasminogen activation and the altered growth kinetics of transformed cells remains equivocal [6, 7]. Previously the growth kinetics of transformed cells has been investigated by the use of indirect methods involving plasmin inhibitors. An alternative approach to the investigation of the link between the growth kinetics of transformed cells and plasminogen activation is to attempt to induce some

of the characteristics of malignant cells in normal cells by treatment with proteases or by co-culture with transformed cells; the products of plasminogen activation by transformed cells being free to act on normal cells when in co-culture. It has been shown that normal cells in co-culture temporarily assume a transformed morphology [4] and we have shown [8] that 3T3 cells (not normally agglutinable) co-cultured with SV40 3T3 cells become agglutinable with concanavalin A (ConA) as a result of plasminogen activation. It is well known that changes in growth kinetics can be induced in normal cells in vitro by mild proteolysis [9, 10, 11], and that their morphology changes to resemble transformed cells [9]. In this paper we investigate the possibility that plasminogen activation by transformed cells initiates cell cycle traverse in co-cultured 3T3 cells. In these experiments the 3T3 cells were first grown to confluent quiescence, since when subconfluent they

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produce significant levels of plasminogen activator [12, 13], while at confluence the level of activator produced by 3T3 cells declines to a low level [5, 13]. We found morphological changes and decreased adhesion to substrate concomitant with stimulation of DNA synthesis when 3T3 cells were co-cultured with their transformed counterparts. Our results suggest that plasminogen activation initiates DNA synthesis by enhancing serum stimulation.

MATERIALS AND METHODS

Cell cultures and co-culture

Swiss mouse 3T3 cells and their transformed equivalents SV40 3T3 were routinely cultured in Dulbecco's Minimal Essential Medium (DMEM) and 10% fetal calf serum (FCS) except under varying conditions described below. The process of co-culture in which 3T3 cells and SV40 3T3 cells are grown in the same medium, but isolated on separate coverslips, and the fact that under these conditions SV40 3T3 cells, but not 3T3 cells, produced detectable plasminogen activator, have been described previously [8].

In experiments designed to minimise serum stimulation the cells were seeded at $1.5 \times 10^4/cm^2$ and brought to quiescence by incubating for 3 days in 0.5% FCS. They were subsequently co-cultured for 18 h in their original medium to which [³H]TdR (10 000 mCi/mmol) had been added to a final concentration of 2 μCi/ml. Lyophilised pig blood plasminogen (Sigma) at 20 μg/ml was also added to some groups at the onset of co-culture. The 3T3 cells were then fixed in fresh methanol/acetic acid (3:1) for 10 min, washed for 2 h in running water and processed for autoradiography.

In a modification of these experiments substituting normal serum levels for added plasminogen during co-culture, 3T3 cells were seeded at $1 \times 10^4/cm^2$ and grown for 5 days in 0.5% calf serum (CS) to produce a subconfluent monolayer. At the time of co-culture fresh medium +10% CS was added. After 19 h cultures were pulsed with 2 μCi/ml [³H]TdR for 1 h. They were then washed with phosphate-buffered saline (PBS), fixed in 10% trichloroacetic acid (TCA) and then washed for 20 min in distilled water, airdried, and counted on a scintillation counter.

In a third set of experiments 3T3 cells were seeded at $2 \times 10^5/cm^2$ and grown for 6 days to confluent quiescence in 10% FCS without a change of medium. They were then co-cultured with equal numbers of SV40 3T3 cells in 10% CS. At 24 h intervals from the time of co-culture the cells were pulsed with 2.0 μCi/ml [³H]TdR (22 000 mCi/mmol) for 1 h. They were then processed for scintillation counting as described previously.

In order to confirm that similar effects to those attributed to plasminogen activation could be achieved in a plasminogen-free system in the presence of added

plasmin, 3T3 cells were grown to confluence in 10% CS from which plasminogen had been removed by affinity chromatography on a lysine–Sepharose column. The medium was then changed for fresh medium containing either 1.08, 5.11 or 17.16% of plasminogen-free CS, previously held at pH 3 for 15 min to remove potential plasmin inhibitors, to which was added [³H]TdR at 7.0 μCi/ml. Human plasmin (AB Kabi, Stockholm) was added to some groups at 0.16, 0.8 or 2 casein U/ml. After 24 h TCA precipitates were prepared and counted on a scintillation counter.

Autoradiography

The coverslips were coated with Ilford L4 and after exposure and staining in Giemsa, 500 cells were examined in each experimental group. Nuclei with grain counts higher than background were counted as positive.

Trypsin-sensitivity test

The time required for cells to round up on their substrate during trypsin treatment was used to detect whether plasmin-mediated proteolysis had occurred in co-culture. The assay was carried out according to the method previously described [14].

Scanning Electron Microscopy (SEM)

The 3T3 cells were seeded on 13 mm diameter coverslips at $1 \times 10^3/cm^2$ (low density) and $2 \times 10^5/cm^2$ (high density). They were cultured in DMEM+10% FCS overnight in 5% CO_2/air at 37°C. They were then mono- or co-cultured for 3 days in fresh medium and processed for SEM as follows. The coverslips were washed once in unsupplemented medium, fixed for 1 h in DMEM+2% glutaraldehyde, washed in PBS for 30 min. They were then fixed in OsO_4 for 1 h, dehydrated and critical point dried.

Planimetry

The cells were photographed under phase contrast and enlargements of known size made. The areas of the cells on these photographs were then measured with a planimeter and the actual area of cell spread was calculated from measurements made on 50 cells.

RESULTS

The initiation of DNA synthesis in quiescent 3T3 cells in co-culture with SV40 3T3 cells

The effect of co-culture on 3T3 cells was investigated by monitoring changes in [³H]-TdR incorporation under a variety of experimental conditions. Since serum stimulates thymidine incorporation and cell division when added to quiescent cultures [15],

Table 1. [³H]TdR incorporation into nuclei by 3T3 cells in 0.5 % FCS after 18 h in co-culture with SV40 3T3 cells

Culture conditions	Plas-minogen	% labelled nuclei	P value
Monoculture	−	2.1±0.9	−
Monoculture	+	2.8±1.3	>0.1
Co-culture	−	3.5±0.8	>0.1
Co-culture	+	9.7±0.9	<0.001

The techniques used for co-culture have been previously described [8]. Lyophylized pig blood plasminogen (Sigma; 2.5 U/mg was added at 20 μg/ml). 3T3 cells retained in monoculture were grown at the same cell-to-medium ratio as the total cell population in co-culture. Five groups of 100 nuclei were examined to obtain each count and were considered positive if labelling exceeded background levels.

Fig. 1. *Abscissa:* no. of days for which 3T3 cells were co-cultured with SV40 3T3 cells; *ordinate:* [³H]TdR uptake (cpm×10⁻⁴) by 3T3 cells.

[³H]TdR incorporation by 3T3 cells in high serum during 4 days in co-culture with SV40 3T3 cells. Cultures were pulsed with 2 μCi/ml [³H]TdR for 1 h and TCA precipitates were counted. Each point is a mean of 8 samples; data for days 1–4 is presented as least squares regression line plots. On all days the rates of uptake in co-culture (●—●) are significantly higher than in monoculture (○—○) ($P<0.001$). Over this period the rate of decline of label incorporation was exponential ($r=0.98$, $P<0.001$ in each case) and was significantly faster ($P<0.002$) in monoculture ($b=0.901$) than in co-culture ($b=0.691$). The plots have been extrapolated (---) to demonstrate the theoretical extinction of the difference between co-culture and monoculture when serum stimulation is increased.

three types of experiments were undertaken using different serum concentrations and cell densities in order to distinguish between the effects of serum stimulation and plasminogen activation on [³H]TdR incorporation, and to investigate the relationship between these two factors. (1) In order to minimise serum effects cells were seeded at low density in Dulbecco's medium (DMEM) with 0.5 % FCS to produce a quiescent subconfluent population, and co-culture with SV40 3T3 cells was then carried out in a similar fresh medium. Because plasminogen may have become limiting in 0.5 % serum it was added to some groups. The [³H]TdR nuclear labelling index for the 3T3 cells was measured in autoradiographs after 18 h in co-culture. Previous experiments on the growth of 3T3 cells brought to quiescence in 0.5 % serum had shown that the peak of S phase occurred at 18 h following growth stimulation. A five-fold increase in nuclear labelling index occurred in co-cultured 3T3 cells, but only in the presence of added plasminogen (table 1). This indicates that a combination of plasminogen and SV40 3T3 cells, which activate the plas-
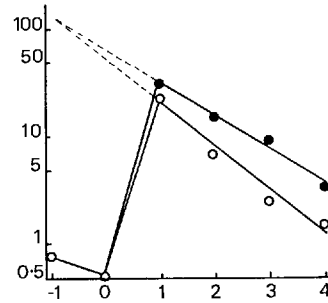
minogen [6, 8], stimulates quiescent subconfluent cells to synthesise DNA. (2) The [³H]TdR incorporation into 3T3 cells grown to subconfluent quiescence in 0.5 % calf serum but without added plasminogen over a 5 day period was investigated. Prior to co-culture incorporation was 301±68 cpm [³H]TdR/cm². Eighteen hours after a change of medium to 10 % CS 3T3 cells in monoculture incorporated 1 462±87 cpm [³H]TdR/cm² compared with 3T3 cells in co-culture which had reached levels of incorporation of 2 025±122 cpm [³H]TdR/cm² ($P<0.01$). This experiment indicates that the addition of plasminogen alone is not a cause of DNA synthesis, as both mono- and co-culture had plasminogen available from the serum. However, the relationship between the effects of plasminogen activation and serum stimulation remain equivocal at this stage.
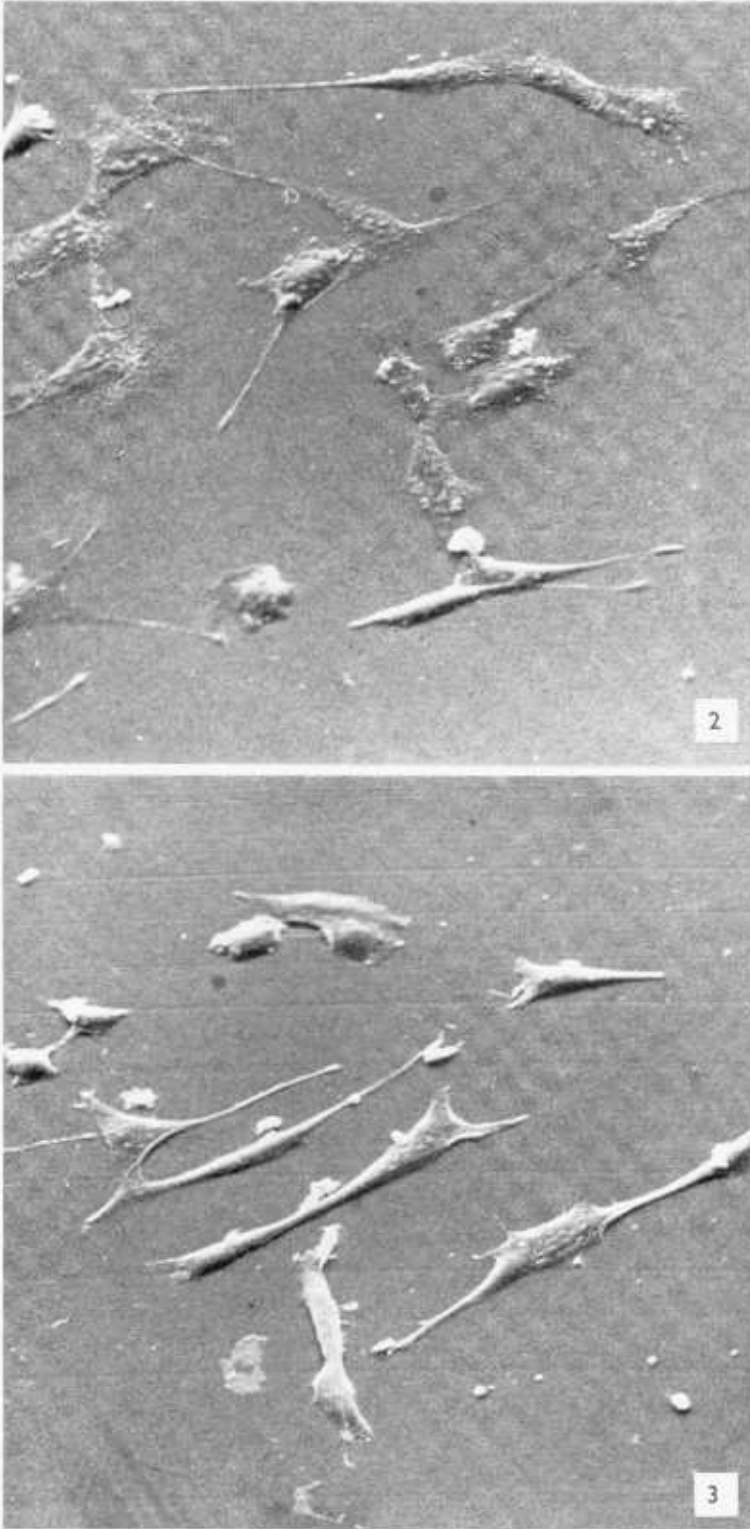
Figs 2–5. The effect of co-culture on the morphology of 3T3 cells. 3T3 cells were seeded at low (figs 2, 3) or high (confluent) density (figs 4, 5) in DMEM+10% FCS, and were grown for 3 days in monoculture (figs 2, 4) or in co-culture with SV40 3T3 cells (figs 3, 5). Subconfluent monocultured cells are more closely apposed to the substrate than they are in co-culture, and at confluence this change in adhesiveness causes fissures to appear in the monolayer. ×1900.
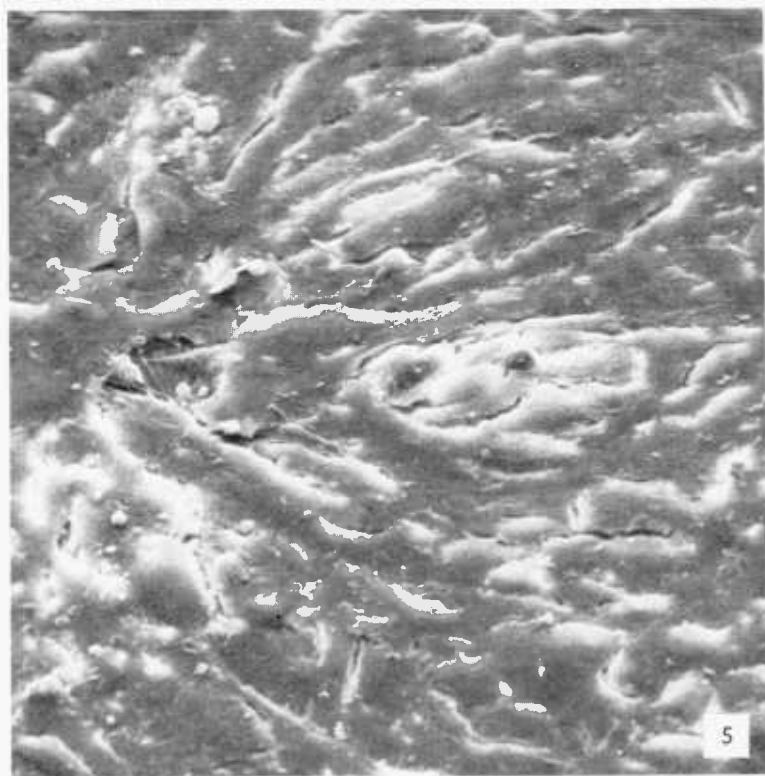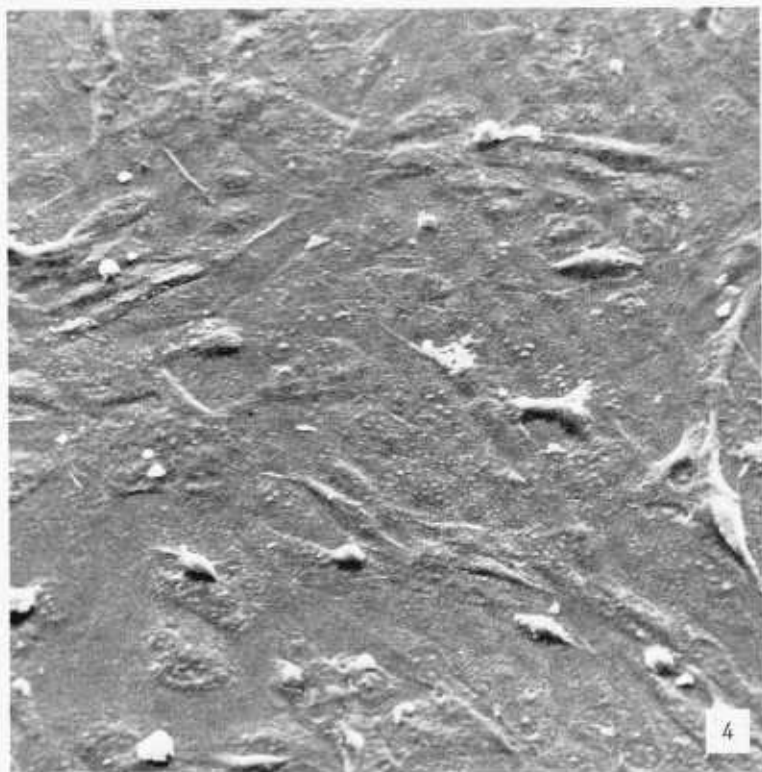
Table 2. *Changes in mean cell area of 3T3 cells over a 3-day period in co-culture with SV40 3T3 cells*

Day of expt	Area of co-cultured 3T3 ($\times 10^{-4}\ \mu m^2$)	Area of mono-cultured 3T3 ($\times 10^{-4}\ \mu m^2$)	P value
0	–	144±12	–
1	81±13	97± 9	<0.1
2	58± 7	81± 8	<0.05
3	52± 4	83± 9	<0.01

We therefore investigated the relationship between plasminogen activation and serum stimulation by monitoring [³H]TdR incorporation into 3T3 cells grown to confluent quiescence in DMEM+10% calf serum over a 5-day period and subsequently co-cultured in fresh medium (fig. 1). After 24 h the incorporation of thymidine increased from a background level of 547±9 cpm to 21769±1115 cpm in monoculture and to significantly higher levels ($P<0.001$) of 30293±394 in co-culture. As serum stimulation declined co-cultured cells incorporated relatively larger amounts of [³H]TdR than did control cells. This result demonstrates that confluent as well as subconfluent co-cultures are susceptible to plasminogen activation-mediated DNA synthesis and that the effect becomes increasingly prominent as the cells return to the quiescent state. In order to provide more convincing evidence that the observed changes were attributable to plasminogen activation, 3T3 cells were grown to confluence in plasminogen-free CS and subsequently challenged with fresh medium containing different levels of fresh plasminogen-free and plasmin inhibitor-free serum, to which a pure preparation of plasmin had been added. We found that in medium containing low (1.08%) levels of serum, the addition of plasmin at 0.16 casein units/ml

increased [³H]TdR uptake from 44372± 7165 dpm to 94727±18722 dpm ($P<0.05$). However at higher serum levels (5.11 and 17.16%) although serum alone significantly increased [³H]TdR incorporation, no extra DNA synthesis was stimulated by the addition of plasmin. Thus plasmin enhanced DNA synthesis in previously quiescent 3T3 cells, but only when serum stimulation was suboptimal.

Changes in morphology and trypsin sensitivity of 3T3 cells in co-culture with SV40 3T3 cells

Protease treatment can cause alterations in normal cell morphology [9], adhesion [16], and anchorage-dependent growth [17] reminiscent of transformed cells; it is possible that 3T3 cells in co-culture assume the morphological [4] and adhesive characteristics of transformed cells. Morphological changes in the 3T3 cells were assessed quantitatively over a 3-day period by planimetry (table 2). The mean cell area of subconfluent populations of 3T3 cells de-
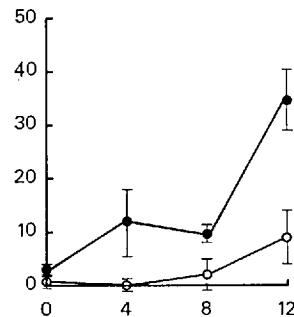
Fig. 6. Abscissa: incubation time (min); *ordinate:* percentage of 3T3 cells rounding up in trypsin solution.
Following 3 days of mono- or co-culture, 3T3 cells on coverslips were placed in 0.025% trypsin (10800 BAEE U/mg) for varying lengths of time from 0–12 min. Photographs taken at each time interval were analysed for the percentage of cells rounded up in each group. Cells in co-culture were significantly more trypsin sensitive. ●—●, Co-culture; ○—○, monoculture; $P<0.05$ at 12 min.

creased, but the decrease was significantly greater in co-cultured cells than in mono-cultured cells (*P*<0.01). These changes were reflected in scanning electron micro-graphs of confluent and subconfluent 3T3 cells (figs 2–5). The greater decrease in cell area in co-culture was due to lateral round-ing and consequent partial loss of close ap-position between cell and substrate (figs 2 and 3). In confluent co-cultured cells this change is associated with the presence of fissures in the cell sheet (figs 4 and 5).

Since in normal cells there is a linear relationship between cell area and trypsin sensitivity [14] we compared the trypsin sensitivity of monocultured with co-cul-tured 3T3 cells, using the technique pre-viously described [14], in order to quantify the degree of loss of adhesion due to plas-minogen activation implied by the decrease in area of co-cultured 3T3 cells described above. On day 3, 39±10% of 3T3 cells in co-culture compared with 9±4% (*P*<0.05) of cells in monoculture rounded up com-pletely after 12 min in 0.025% trypsin (10 800 U/mg) (fig. 6).

DISCUSSION

When 3T3 cells were grown to subcon-fluent quiescence in 0.5% FCS and subse-quently transferred to co-culture with SV40 3T3 cells the number of cells in S phase increased five-fold in the presence of added plasminogen (table 1). These results indi-cate that under conditions of plasminogen activation in which SV40 3T3 activator con-verts added plasminogen to plasmin [8] a proportion of quiescent cells are stimulated to traverse the cell cycle. DNA synthesis also increases in similar experiments where cells are transferred to 10% serum at co-culture to obviate the need for added plas-minogen. Under those physiologically more

normal conditions, however, it is difficult to distinguish the effects of serum stimulation [18] from those of plasminogen activation. In order to investigate the relationship be-tween these two factors a third type of ex-periment was undertaken in which con-fluent quiescent 3T3 cells were serum stim-ulated and simultaneously subjected to plasminogen activation by transferring to co-culture. The relationship between the two was then monitored by observing changes in the rates of thymidine incor-poration as serum stimulation declined over the next few days (fig. 1). This showed that the effect of plasminogen activation became relatively more pronounced as serum stimu-lation declined. Extrapolation from this data indicates a theoretical point at which serum stimulation is maximal and where plasminogen activation has no additional ef-fect. The simplest interpretation of this data is that the effect of plasminogen activa-tion is to enhance the effects of serum stimulation when the latter is suboptimal. This view is supported by our observation that, in the absence of plasminogen, plas-min produces the same effect as plasmino-gen activation, but only when the serum is present in the fresh medium at very low concentration (1%). The fact that plasmin fails to enhance DNA synthesis when the concentration of serum is raised to 5% or more adds weight to our suggestion that plasminogen activation acts by increasing the response of cells to serum growth fac-tors in the medium when these are in short supply.

When normal fibroblasts are deprived of serum they cease growing and accumulate in the G1 stage of the cell cycle. On serum pulse a proportion of cells becomes irre-versibly committed to initiate a round of DNA replication. The committment to enter S phase is a random event of calcul-

able probability [19], the rate constant for transition probability being a function of the duration of the pulse and of serum concentration [20]. The subsequent doubling time of 3T3 cells is not related to serum concentration, but the density at which they become quiescent is [21]. Such quiescent monolayers can be stimulated to divide further by wounding [22] or by disruption of the diffusion boundary layer [23]. In these cases it appears that the end result is to increase the availability of serum growth factors to the cells by disruption of a layer of growth factor-depleted medium overlying the monolayer. In our system plasminogen activation caused rounding up of subconfluent 3T3 cells, associated with a loss in close apposition to the substrate (figs 2 and 3), and these changes may be adequate to disrupt the diffusion boundary layer and allow greater access of growth factors, thus stimulating a new round of DNA synthesis. Alternatively, or additionally, an increase in availability of serum growth factors and subsequent initiation of DNA synthesis could be brought about by a change in cell membrane permeability produced by mild proteolysis [24]. In the case of confluent quiescent monolayers plasminogen activation resulted not only in the rounding up of cells but in the opening of fissures and consequent decrease in cell-to-cell contact; these changes would also result in the greater access of undepleted serum to the cells. Our proposal that DNA synthesis was stimulated by the renewed availability of serum growth factors is supported by the observation that neither plasminogen activation nor plasmin initiated additional cell cycle traverse when serum was in plentiful supply.

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