<u>Giardia intestinalis</u> :

Aerobic Metabolism and Physiology

of <u>in vitro</u> Growth.

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Sally Jayne Miller

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ABSTRACT

G. intestinalis; the causative agent of giardiasis, parasitises a
number of vertebrates including man; and has a worldwide
distribution. Although giardiasis is now a widely recognised public
health concern; little is known of its aetiological agent. This is
primarily due to the fact that a protocol for the routine axenic
cultivation of this intestinal parasite was not available until
1976. With the advent of in vitro cultivation, an increasing number
of reports have outlined the in vitro growth requirements of G.
intestinalis; however; the physiology and unique metabolism of this
protozoan still require further clarification.

Utilising two strains of G. intestinalis (Bris/83/HEPU/106 and
Hast/87/MUGU/68), the influences of environmental factors such as pH
and temperature on axenic culture growth were investigated.
Variations in both temperature and pH were shown to effect the in
vitro growth rate of the two strains examined. Growth of
Bris/83/HEPU/106 was markedly impaired at non-optimal temperature;
(optimal growth of Bris/83/HEPU/106 and Hast/87/MUGU/68 occurred at
$37^{O}C$); while growth of Hast/87/MUGU/68 continued, at a reduced rate,
over a wider, non-optimal temperature range ($30-40^{O}C$). Both strains
exhibited marked pH optima for culture growth (pH 6.75-7.50) with a
rapid decline in culture growth rates outside these pH levels.

Clonal growth of G. intestinalis trophozoites in semi-solid agarose
has been utilised in the past as an assay of trophozoite viability
in vitro. The suitability of such an assay for use during this
study was investigated for both Bris/83/HEPU/106 and
Hast/87/MUGU/68. Over the range of agarose concentrations examined,
the colony forming efficiency (CFE) of both strains was extremely
variable. While Hast/87/MUGU/68 was better adapted to growth in
agarose medium, with CFE of up to 60% recorded; these rates of
clonal growth were often not reproducible, as the growth of
trophozoite colonies remained inconsistent despite duplication of
all assays.

The thiol reducing agent L-cysteine, has been reported to be a specific growth requirement of G. intestinalis in vitro. The correlation between reducing conditions and the growth and attachment of Bris/83/HEPU/106 and Hast/87/MUGU/68 in culture, was investigated as trophozoites were exposed to a range of L-cysteine concentrations in TY1-S-33 growth medium. Enhanced growth of experimental cultures was directly related to increases in L-cysteine concentration and corresponding decreases in the O-R Potential of growth medium. Culture growth occurred at a maximal rate where the concentration of L-cysteine in growth medium exceeded 0.15% w/v. All cultures failed to grow in the absence of L-cysteine.

Trophozoite attachment in culture was most rapid during the 30-90 minutes following culture establishment. Under elevated L-cysteine concentrations (0.15-0.25% w/v) this attachment reached maximal levels (85-95%). In the absence of L-cysteine, attachment of trophozoites in culture continued, but at a markedly reduced rate.

The oxygen sensitivity of G. intestinalis trophozoites was investigated in TY1-S-33 utilising a protocol developed during this study, where the exposure of trophozoites to dissolved oxygen was directly controlled through adjustment of oxygen flow into growth medium. Bris/83/HEPU/106 and Hast/87/MUGU/68 trophozoites displayed a similar degree of oxygen sensitivity at 37°C. A slow decline in culture viability was recorded upon exposure of trophozoites to 4.0-6.0 ppm dissolved oxygen in growth medium. At 8.0 ppm; exponential killing of trophozoites was preceded by a 'lag phase' of 3-4 hours duration. In contrast; the killing of cultures commenced almost immediately after exposure of trophozoites to 12.0 ppm dissolved oxygen. At temperatures below 37°C (20°C and 30°C), Bris/83/HEPU/106 exhibited a reduced sensitivity to elevated dissolved oxygen levels in TY1-S-33, as both the $T_{\frac{1}{2}}$ of killing, and the lag phases preceding this killing were extended. The basis for the observed 'temperature-dependant' oxygen sensitivity of G. intestinalis is not known.

Oxygen consumption by <u>G. intestinalis</u> has recently been reported by several workers; however; there is still very little known of the metabolic role of 'active respiration' in this 'aerotolerant anaerobe'. Consumption of oxygen by Hast/87/MUGU/68 in PBS was demonstrated using a Model 97-08 Oxygen Electrode. Dissolved oxygen was removed from PBS by trophozoites at a rate of 3.2-5.3 10^{-9} ppm/cell/hr. This oxygen consumption was inhibited up to 50% by the flavoantagonist, Quinacrine dihydrochloride, at concentrations of 250-1000 μg/ml in PBS solution. The concentrations of Quinacrine which were inhibitory to oxygen consumption by trophozoites over a 5 hour period were well in excess of the Quinacrine MLC (Minimum Lethal Concentration).

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CHAPTER ONE : INTRODUCTION

1.1   The Organism

    1.1.1   Structure and Taxonomy

Giardia intestinalis is a flagellate within the order Diplomonadida. Members of the genus Giardia are parasitic of a wide range of vertebrates. G. intestinalis is of interest as a parasite of man.

Giardia species have a diphasic life cycle which includes a binucleate flagellated trophozoite stage, and a quadrinucleate resistant cyst stage.

Flagellates of the genus Giardia were first described by Anton van Leeuwenhoek in 1681 during microscopic observations of his own faeces during a diarrhoea attack. These observations implicated an association between the parasite and intestinal disease. Lambl (1859) and Grassi (1879) (see Filice, 1952) 'rediscovered' the genus and made the first formal descriptions of the trophozoite and cyst.

G. intestinalis trophozoites are 12-15 μm long x 6-8 μm wide; pear-shaped, and bilaterally symmetrical. (Filice, 1952).

Trophozoites are dorsally convex and ventrally concave. The anterior of the ventral surface is occupied by an ovoid 'sucking disc' (Filice, 1952; Cheissin, 1964; Friend, 1966; Holberton, 1973). This feature; along with dorso-ventral flattening differentiates Giardia from other Diplomonads. (Feely et al., 1984)

The ventral sucking disc is a 'cytoskeleton of microfilaments and microtubules in association with other Filamentous structures'. (Feely et al., 1984). Two median bodies lie transversely across the middle of the trophozoite. (Simon (1921), see Filice, 1952). These median bodies are composed largely of microtubules. (Friend, 1966). Their function

remains obscure, though it has been suggested that they may serve as a depot of cytoskeletal proteins for mobilisation prior to mitosis or excystation. (Feely et al., 1984).

Trophozoite motility is facilitated by four pairs of flagella. (Simon (1921); Hegner (1922b); Kofoid and Swezy (1922), see Filice, 1952). These flagella were assumed to beat rapidly as the trophozoite moved with an 'up and down motion' likened to 'skipping'. (Cunningham (1881); Dobell (1920), see Filice, (1952). Glebski (1967), determined that flagella did not beat rhythmically, and so their contribution to the forward movement of trophozoites was small. Oarlike movements of anterior and posterior-lateral flagella, coupled with dorsal or lateral flexion of the trophozoite tail which may act as a rudder; are thought to be responsible for the rotational forward movement observed. (Glebski, 1967; Holberton, 1977). Flagella axonemes have been determined to have the typical '9 + 2' arrangement common to eukaryotic flagella. (Feely and Erlandsen, 1982).

No structures resembling mitochondria have been detected in Giardia trophozoites. (Friend, 1966). Vacuoles (100-500 nm) observed adjacent to the plasmalemma over the trophozoite body were thought to be modified mitochondria (Cheissin, 1965), but a demonstration that these vacuoles have the ability to incorporate exogenous ferritin; and the detection of acid phosphatase products in the vacuoles; indicated they have a lysosomal function. (Bockman and Winborn, 1968; Soloviev, 1968; Nemanic et al., 1979; Lindmark, 1980; Radalescu et al., 1982; Feely and Dyer, 1987).

G. intestinalis cysts are elliptically shaped and range in size from 6-10 μm. The cyst wall is hyaline and composed of fibrillar elements and fine particles. It varies in thickness from 0.3-0.5 μm, has a fibrous outer wall and an electron-dense inner membrane. (Filice, 1952; Sheffield and Bjorvatin, 1977; Nemanic et al., 1979; Tombes, et al., 1979; Luchtel et al., 1980; Coggins and Schaefer, 1984, 1986). It is suggested that the cyst wall is produced from a system of vacuoles and tubules

beneath the cell membrane of trophozoites. (Friend, 1966). Most organelles prominent in the cytoplasm of the trophozoite stage are also present in the cyst, ie nuclei, karyosomes, median bodies and flagellar axonemes are all visible by light microscopy. Flagella and the adhesive disc are absent from mature cysts. (Sheffield and Bjorvatin, 1979).

1.1.2   Speciation

The genus Giardia has been divided into species on the basis of gross morphometrical differences, and assumptions of rigid host specificity. Using these criteria, over 40 species of Giardia have been differentiated (Hegner (1922b) see Filice, 1952; Abraham, 1962; Solovjev (1975) see Meyer and Radalescu, 1979; Kulda and Nohynkova, 1978). Several workers have questioned the statistical validity of morphometric comparisons, and the host specificity of trophozoites has been disputed after observation of some degree of cross-infectivity. (Roberts-Thomson et al., 1976a; Grant and Woo, 1978; Davies and Hibler 1979; Meyer and Radalescu, 1979; Dykes et al., 1980; Hewlett et al., 1982; Bertram et al., 1984).

Filice (1952) divided the genus into three morphological groups on the basis of median body morphology which he stated as the only reliable characters for differentiation.

(a) G. agliis group:- Median bodies fused into a single, club-shaped rod, parallel to the long axis of the trophozoite.

   -   Narrow elongate body

   -   Found in amphibians, typically disappears after metamorphasis of the host.

(b) G. intestinalis group: - Median bodies lie approximately across the body of the trophozoite

   - Body outline is pyriform

   - Found in man, other mammals, some birds and reptiles.

(c) <u>G. muri group</u>: - Rounded median bodies parallel to the
                               long axis of the trophozoite
                          -    Body is short and broad
                          -    Found in rodents and birds.

Median body morphology has also been used to differentiate individual species. (Nieschulz (1924), see Filice, 1952; Grant and Woo, 1978a).

Most recently, chemotaxonomy, including comparisons of trophozoite isoenzymes by starch gel electrophoresis (SGE) and polyacrylamide gel electrophoresis (PAGE), has been used to differentiate animal and human isolates. Isolates have been divided into three zymodemes on the basis of the comparisons. (Woo, 1984; Korman <u>et al</u>., 1986).

1.1.3   Excystation and Encystment

The process of excystation has only been seen and described this century. Several early reports described <u>in vivo</u> excystation occurring in faeces and intestines of hosts (Hegner (1925a, 1927c), see Filice, 1952). A partial <u>in vitro</u> excystation of <u>Giardia</u> cysts at 37°C has also been reported (Hegner (1927c), see Filice, 1952).

During excystation there is considerable movement in the cyst, and the cyst wall ruptures at the pole opposite the nuclei. After release, the trophozoite undergoes cytokinesis and a typical trophozoite morphology is seen. The trophozoite then attaches rapidly to the available substratum. (Filice, 1952; Bingham <u>et al</u>., 1979; Feely <u>et al</u>., 1984; Buchel <u>et al</u>., 1987).

By exposing cysts to an induction medium of pH 2.0 and a regime of washing and incubation in physiological saline or excystation media; <u>in vitro</u> excystation is now routinely possible using cysts from a variety of animal sources. (Bingham <u>et al</u>., 1979; Rice and Schaefer, 1981a; Schaefer <u>et</u>

al., 1984; Kaur et al., 1986; Isaac Renton et al., 1986;
Kasprzak and Majewska, 1985; Feely, 1986).

Ability to excyst _in vitro_ is now a preferred method for
assaying viability of trophozoites after they have been exposed
to a range of storage conditions, or to 'cyst inactivating
agents' such as chlorine or other disinfectants (Bingham et
al., 1979; Hibler et al., 1980; Jarrol et al., 1981; Rice and
Hoff, 1981; Hoff et al., 1985).

Although observed and described by several workers _in situ_
(Schaudin (1903); Rodenwaldt (1912); Lavier (1935, 1942) see
Filice, 1952). There have been no reports of successful _in
vitro_ induction of encystment.

1.1.4   Cell Division

Giardia species reproduce asexually through a process of binary
fission. Cytokinesis was thought to occur only in the cyst,
(Wenyon, 1926), until trophozoite division was described.
(Kofoid and Christiansen, 1915b; Lavier (1939), see Filice,
1952).

During trophozoite division, karyokinesis occurs first,
followed by division of the locomotor apparatus, the adhesive
disc, and finally, the cytoplasm. During cyst formation,
fission of the cytoplasm does not occur. (Coggins and
Schaefer, 1984; Buchel et al., 1987).

1.2   Trophozoite Attachment and Detachment
1.2.1   Attachment _in vitro_ and _in vivo_

Muller (1890) and Zebel (1902) (see Filice, 1952) first
described a 'sucker-like organ' on _Giardia_ trophozoites for
adherence to apical surfaces of epithelial cells. This was
assumed to be the only purpose of the adhesive disc. (Kofoid
and Swezy, (1922), see Filice, 1952). Since there was no
evidence for any other function. After observation of

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rhythmical contractions of the sucking disc, it was proposed
that trophozoite attachment was due to the 'grasping nature' of
the disc. (Lambl (1859); Simon (1921), see Filice, 1952).

Friend (1966) suggested, on morphological grounds; that
adhesion was due to contractile or grasping action of a ventro-
lateral flange, with the adhesive disc acting as a support or
pontoon. This theory did not explain the need for close
contact of the disc to the host cell surface. (Erlandsen and
Feely, 1984). Holberton (1973, 1974) proposed a system where
beating of ventral flagella produced a suction pressure up to
10^2 dynes/cm^2 due to movement of media through marginal and
central grooves. Contraction of the ventro-lateral flange was
said to determine the magnitude of suction pressure, and so
control attachment and detachment. Holberton, assumed that
ventral flagella emerge from the disc centre (Erlandsen and
Feely, 1984). Numerous S.E.M. studies have shown they are, in
fact; caudal and dorsal to the posterior edge of the disc.
(Erlandsen, 1974; Erlandsen and Chase, 1974; Mueller et al.,
1974; Owen et al., 1979; Feely et al., 1982). Also, fixed
trophozoites with inactive flagella are able to remain
attached. (Mueller et al., 1974; Owen et al., 1979; Feely and
Erlandsen, 1981; Feely et al., 1982). Kosjuk (1973) suggested
that a central pair of flagella act as a pump with a valve
arrangement for suction and adhesion. After noting wave-like
propulsions of flagella, and the suggestion that fluid is
removed from under the flattened disc to produce a negative or
reduced pressure; Soloviev (1968) (see Erlandsen and Feely,
1984) modified this theory to include the arching of the
ventral disc to create a chamber between the disc and the host
cell, which is open at anterior and posterior ends. This
chamber would then be exposed to negative pressure produced by
the flagella. No account of regulation of this system was
provided.

Microtubules in the adhesive disc are arranged in a spiral
conformation. Mueller et al., (1974), proposed that if a
coiling/uncoiling interaction was possible between

microtubules, the resultant change in disc diameter would lead to a grasping action at the edge of the disc. Since microtubular arrays are separated by dorsal ribbon arrangements, this theory was discounted. (Holberton, 1973).

Lesions caused by attachment of Giardia trophozoites to intestinal epithelia are mirror-images of the adhesive disc, indicating a close contact between the disc and epithelial cells. (Takano and Yardley, 1965; Friend, 1966; Holberton, 1973; Erlandsen, 1974; Erlandsen and Chase, 1974).

From interference-reflexion microscopy; all three morphological groups of Giardia have the same patterns of contact with the substratum. (Erlandsen and Feely, 1981). Using SDS gel electrophoresis, Feely et al., (1982), found evidence of actin, α actinin, myosin and tropomyosin in the disc periphery. It was suggested these contractile proteins have a role in disc adherence to epithelial cells. (Holberton, 1973). Presence of cytochalasin B or a reduction in Ca^{2+} levels decreases trophozoite attachment to substrata. (Feely et al., 1982).

From these findings, Erlandsen and Feely (1984) proposed the following mechanism for trophozoite attachment:
(i)   trophozoite with flat disc comes to cell surface
(ii) Filaments in the lateral crest contract
(ii) Ventral disc buckles due to a change in disc diameter.
(iv) There is either a reduced pressure under the disc, or an undefined 'adhesive interaction' between the lateral crest and the substratum.

1.2.2   Trophozoite Detachment

Trophozoite detachment is initiated with a large dorsal tail flexion which releases negative pressure under the ventral disc (Erlandsen et al., 1978). The driving force for this dorsal flexion may be the caudal flagella. Once the trophozoite is free of the substratum; further separation takes place in steps

due to flagellar activity. (Erlandsen and Feely, 1984).
The role of contractile proteins in the process of detachment
is not known. (Glebski, 1967; Feely et al., 1982).

1.3   Giardiasis

1.3.1   Giardia as a Pathogen

G. intestinalis is the aetiologic agent of giardiasis; a
syndrome which encompasses a range of conditions from
asymptomatic carriage, to chronic diarrhoeic malabsorption.

Until it was proposed that Giardia sometimes causes diarrhoea
in man, (Grassi (1881); Grassi et al., (1888); Moritz et al.,
(1892); Fantham et al., (1916), see Filice, 1952), the
flagellate was thought to be a harmless commensal since it was
often found in healthy individuals (Wenyon, 1915).

Confirmation of the pathogenic nature of G. intestinalis came
with the observed reversal of clinical manifestations of
giardiasis after eradication of the parasite with specific
treatment. (Veghelyi, 1939; Alp and Hislop, 1969; Moore et
al., 1969). Brown (1948) and Ormiston et al., (1942) made the
first full descriptions of the diarrhoeal syndrome and
suggested that faecal contamination was a factor in
transmission of the infection.

1.3.2   Pathogenesis

Transmission of giardiasis is facilitated by the diphasic life
cycle of Giardia. Trophozoites colonising the jejunum of the
host, encyst as they are passed in dehydrating faeces through
the distal small intestine and colon. (Owen et al., 1979).
These cysts remain viable in moist faeces until they are
ingested, at which stage excystation occurs in the duodenum,
and the cycle of trophozoite colonisation recurs in a new host.

Asymptomatic cyst passage is common. The factors responsible
for converting an asymptomatic infection into a symptomatic

infection are inherent in the host, not the organism. An infection is more likely to be symptomatic in a host with reduced gastric acidity. (Yardley et al., 1964).

The median prepatent period of symptomatic giardiasis is usually 14 days, though this period may often last less than one week. (Jokipii and Jokipii, 1977). Acute giardiasis usually lasts for 3-4 days and is characterised by a range of symptoms including nausea, anorexia and low grade fever; which are followed by an explosive diarrhoea with watery, foul-smelling stools, flatulence, abdominal distension and cramps. Blood and mucous are only rarely found in stools. (Moore et al., 1969; Walzer et al., 1971; Andersson et al., 1972; Shaw et al., 1977; Wolfe, 1978; Dykes et al., 1980; Osterholm et al., 1981; Solomons, 1982).

The acute infection usually resolves spontaneously and is followed by either a subacute or chronic stage, in which diarrhoea symptoms persist moderately and fatigue, anorexia and nausea are common. (Wolfe, 1978; Chester, 1985). Prolonged infections also lead to various types of malabsorption including steatorrhea, vitamin B12 malabsorption, disaccharidase deficiency, hypocarotinemia, low serum folate levels and protein-losing enteropathy. (Hoskins et al., 1967; Wright et al., 1977; Wolfe, 1978; Hartong et al., 1979; Solomons, 1982).

During the course of infection there is dense trophozoite colonisation of the jejunal epithelium with adherence to columnar epithelial cells at the base of villi in the proximal 25% of the small intestine (Owen et al., 1979). Damage to the microvillus border of epithelial cells during colonisation could lead to the deficiencies and nutrient malabsorptions discussed earlier. (Wolfe, 1978); but whether such mucosal damage occurs is under dispute. It has been suggested that malabsorption may be the result of some 'direct toxic effect' by the trophozoite. (Alp and Hislop, 1969).

Several workers have been successful in establishing animal models for giardiasis using rats and mice. (Roberts-Thomson et al., 1976 a, b, c; Roberts-Thomson and Mitchell, 1978, 1979; Seghal et al., 1976; Stevens et al., 1978; Owen et al., 1979; Vinyak et al., 1979; Hill et al., 1983). These animal models have been used to study the nature of both the course of infection; and the hosts immune responses to the infection. (Owen et al., 1981; Radalescu and Meyer, 1981; Heyworth 1986; Snider and Underdown, 1986).

1.3.3 Immunology

In addition to the spontaneous resolution of many cases of giardiasis, workers have noted acquired resistance to infection in those individuals repeatedly exposed to Giardia. This is true for both humans and animals. (Moore et al., 1969; Barbour et al., 1976; Roberts-Thomson et al., 1976; Roberts-Thomson and Mitchell, 1978, 1979).

The host immune response to Giardia involves both humoral and cell-mediated reactions. Both mechanisms said to be 'essential for parasite expulsion'. (Ament et al., 1973; Meyers et al., 1977; Owen, 1980). Antibodies to trophozoite antigens have been detected in both the serum (IgG), and intestinal secretions (IgA) of humans and animal models. (Radalescu et al., 1976; Ridley and Ridley, 1976; Visvesvara et al., 1980; Roberts-Thomson and Anders, 1981; Smith et al., 1981, 1982a; Anders et al., 1982; Einfeld and Stibbs, 1984; Heyworth, 1986; Snider and Underdown, 1986). T-lymphocytes, macrophages and mast cells are involved in the cell-mediated response. (Meyers et al., 1977; Roberts-Thomson and Mitchell, 1978; Owen et al., 1981; Radalescu and Meyer, 1981; Smith et al., 1981; Smith et al., 1983a; Heyworth, 1986). During the course of chronic giardiasis, the host produces a normal IgG, IgA and lymphocyte response, but a reduced monocyte and macrophage killing. (Smith et al., 1982).

The importance of elucidating the host immune response to

Giardia infection is twofold. Firstly, the recent isolation and characterisation of a Giardia specific antigen (GSA) is a step towards identifying the Giardia immunogen important in man; (Einfeld and Stibbs, 1984; Edson et al., 1986; Rosoff and Stibbs, 1986), and secondly, there is the potential for use of the antigenic make-up of trophozoites as a criterion for classification. (Moore et al., 1982; Korman et al., 1986).

1.3.4   Diagnosis

The most commonly used method for the diagnosis of giardiasis is direct faecal examination. Repetition of these examinations is essential since cyst excretion can often be variable and therefore undetected for some time. (Danciger and Lopez, 1975; Jokipii and Jokipii, 1977; Markell and Quinn, 1977; Wolfe, 1978; Zimmer and Burrington, 1986). Small bowel biopsies and duodenal fluid examinations are used as a supplement to faecal examination. (Ament, 1972; Madanogopalan et al., 1975; Wolfe, 1978; Gordts et al., 1986). Diagnosis by serological methods including ELISA, immunofluorescence, radioassays, and counterimmunoelectrophoresis is also now feasible. (Visvesvara 1980; Anders et al., 1982; Craft and Nelson, 1982; Thornton et al., 1983).

1.3.5   Epidemiology

Outbreaks of giardiasis occur worldwide with high risk areas being, Asia, West and Central Africa, Mexico, Korea and South-West South America. Prevalence may be up to 30% in some areas, dependant on local sanitary standards. In most countries, children aged 1-5 years are commonly effected. (Kulda and Nohynkova, 1978; Wolfe, 1978; WHO, 1980).

Up to 80% incidence of giardiasis has been reported in persons with hypogammaglobulinemia or other gastrointestinal immunodeficiency syndromes. (Ament and Rubin, 1972).

Most community outbreaks of giardiasis reported in the

literature have been waterborne with infective cysts being maintained in untreated or insufficiently treated water supplies. (Moore et al., 1969; Shaw et al., 1977; Dykes et al., 1980; Lopez et al., 1980; Craun, 1984).

Numerous foodborne outbreaks have also been reported after accidental contamination of food has occurred in doubtful sanitary conditions, or when food handlers were infected with Giardia. Ingestion of contaminated food has resulted in the establishment of infections in many individuals. This foodborne transmission of infection has been repeated in the laboratory using mice as animal models. (Ganganosa and Donadio, 1970; De Carneri and Trane, 1978; Osterholm et al., 1981).

A range of animals including beavers, domestic pets; and even cockroaches; have been implicated as reservoirs or vehicles for the transmission of giardiasis, indicating the zoonotic nature of the infection. *(Davis and Hibler, 1979; Dykes et al., 1980; Hiral et al., 1980; Box, 1981; Kasprzak and Majewska, 1981; Owen, 1984). Transmission of giardia may also occur through sexual activity. (Meyers et al., 1977; Owen, 1984).

1.3.6   Water Treatment

Current treatment of municipal water supplies includes in lime filtration as well as chlorination and UV radiation which have been shown to reduce the viability of Giardia cysts. (Hoff, 1978; Hibler et al., 1980; Jarrol et al., 1981; Rice et al., 1982; Hoff et al., 1984). A number of filter systems may be applied for removal of cysts from water supplies. These include granular medium filters (Logsdon et al., 1981), and slow sand filters (Bellamy et al., (1983) see Logsdon et al., 1984). In addition, a multiple barrier system which includes a combination of filtration and disinfection techniques may be applied. (Logsdon et al., 1984).

Filtration is also used constantly to detect cysts in sewage

effluent, and to quantitate cyst levels in water supplies. (Spaulding et al., 1983; McHarry, 1984).

1.3.7 Treatment of giardiasis, and Sensitivity of G. intestinalis to Drugs in vitro.

Treatment of giardiasis is effected using four antiprotozoal drugs: Metronidazole (flagyl), Tinidazole, Furazolidone and Quinacrine, in a regime which involves the administration of 100-400 mg doses of the drug three times daily for seven days, or; in the case of Metronidazole; the administration of a single 'hammer dose'. (Wolfe, 1978).

Measurements of the activity of these agents against G. intestinalis in vitro (using clonal growth, 3H-Tdr incorporation and trophozoite mobility as measures of trophozoite viability) defined a range of minimum lethal concentrations for each drug: Metronidazole MLC = 0.91-292.05 μmoles/l; Tinidazole MLC = 0.12-50.6 μmoles/l; Furazolidone MLC = 0.43-2.5 μmoles/l; Quinacrine MLC = 0.021-4.02 μmoles/l. (Jokipii and Jokipii, 1980; Gillin and Diamond, 1981; Smith et al., 1982; Boreham et al., 1984).

Use of imidazole drugs in vitro for treatment of giardiasis is tempered with the knowledge that many of these agents are carcinogenic or mutagenic, and it seems that no ideal treatment for giardiasis exists. Quinacrine and Furazolidone have been shown to produce side effects in patients. Adverse reactions resulting from treatment include toxic psychosis, vomiting, fever and a range of hypersensitivity reactions. (Wolfe, 1978; Craft et al., 1981). There is some dispute over the effectiveness of Metronidazole as a chemotherapeutic agent when compared to Quinacrine and Furazolidone, but Meronidazole is generally considered to be the drug of choice, since while it is still a reasonably effective treatment for giardiasis, the side effects subsequent to drug administration are mild, involving only a dark discolourisation of urine, and some degree of nausea, cramps and dizziness. (Bassily et al., 1970;

Madanogopalan et al., 1975; Singh, 1977; Wright et al., 1977; Jokipii and Jokipii, 1978; Wolfe, 1978).

The issue of treatment of giardiasis has been further complicated by the recent suggestion that 'biological variants' of G. intestinalis exist in humans, and that these variations may account for both the variable clinical manifestations of giardiasis, and the failure of some treatments. (McIntyre et al., 1986).

1.4   Axenic Cultivation

From a desire to study G. intestinalis; came attempts to cultivate the organism. Initially, attempts were made to culture Giardia in physiological saline solutions, either *axenically; or with Trichomonads. (Chatterjee, 1927; Penso, 1929). Trophozoite survival was limited in simple media; cultures being maintained for up to 19 days on pig-liver bouillon mixtures. (Poindexter (1932) see Filice, 1952; Iwata and Araki (1960) see Meyer and Radalescu, 1984). Karapetyan (1960), maintained trophozoites symbiotically with C. guilleromondii in a medium containing inactivated human serum, chick embryo extract, tryptic meat digest and Hanks or Earle's solution supplemented with chick fibroblasts which were gradually destroyed. One third of the medium only was exchanged daily.

This was the first culture method that could be repeated by other workers. (Soloviev (1962), see Meyer and Radalescu, 1979). Monoexenic culture of Giardia was continued with either S. cerevisiae or C. guilleromondii as symbionts in media without chick fibroblasts. Death of cultures occurred if the yeast symbiont was removed. (Karapetyan, 1962b, 1963).

By modifying Karapetyan's method to include: addition of yeast daily; intestinal fungus as an additional symbiont; and 25% human serum instead of 10% serum; Giardia trophozoites from rabbit, cat, chinchilla and human sources could be maintained indefinitely. (Meyer and Pope, 1965; Soloviev (1966); Soloviev et al., (1971), see

Meyer and Radalescu, 1979).

Meyer (1970) maintained Giardia from chinchilla and cat sources with yeast, in Karapetyan's medium (M-1). After transfer to a U-tube containing M-3 (a tissue culture medium enriched with yeast extract and reducing agents) trophozoites were separated from yeast by virtue of their mobility. Trophozoites isolated in this way were not able to multiply in M-3 with yeast extract; but were cultured successfully in the same medium after addition of a dialysis bag containing viable yeast. The generation time of G. intestinalis in this medium was estimated to be 18.1 hours. (Danciger and Meyer, 1971). Meyer's M-3 medium, with the addition of agar in 1.0-1.5% concentrations was used to establish 13 axenic strains of G. intestinalis. (Teras and Lakhonina, (1975); Lakhonina et al., (1976); Lakhonina (1978) see Meyer and Radalescu, 1984). Iyer and Gaitonde (1975a, b, 1976), (see Meyer and Radalescu, 1979) maintained trophozoites for 13 days in Pavlov's medium containing saline, yeast extract, horse serum and rice starch. Bacterial associates were said to be necessary since culture supernatants and filtered bacterial sonicates failed to promote growth of Giardia cultures.

Meyer (1976) was the first to report the axenic culture of G. intestinalis. Cultures were established in HSP-2 in the presence of the host's intestinal fungi. (HSP-2 is a variant of the phytone-peptone medium HSP-1). After isolation of trophozoites using the U-tube system; gradual replacement of small volumes of medium removed all yeast, and trophozoites continued to grow under the axenic conditions imposed.

With a view towards suitability of trophozoites for immunological tests; human serum in HSP-2 was replaced by bovine serum. (Visvesvara et al., 1977). Trophozoites from human and monkey sources were maintained for up to 7 months in this medium. (Bingham and Meyer, 1979).

Two media; TPS-1 and TY1-S-33, developed for culture of E. Histolytica (Diamond, 1968; Diamond et al., 1978); were shown to

support growth of G. intestinalis. (Bhatia and Warhurst, 1981; Kasprzak and Majewska, 1983). TY1-S-33 has since been the medium of choice.

A number of modifications have been made to enhance growth of G. intestinalis trophozoites in TY1-S-33, these modifications include the substitution of bovine serum for horse serum; filter sterilization of TY1-S-33 as opposed to autoclaving, and supplementation of the medium with L-cysteine (0.1-0.2%) and bile or bile salts. (Visvesvara, 1980; Belosovic et al., 1982; Farthing et al., 1983; Keister, 1983; Kasprzak and Majewska, 1983, 1985). Most recently; TY1-S-33 has included an extract of whole bovine calf blood (CLEX). As a substitute for foetal bovine serum, the extract is adequate and less expensive. (Wallis and Wallis, 1986). Although most workers now use TY1-S-33 in routine culture maintenance, TPS-1 is still preferred by some workers. (Gordts et al., 1984).

Established axenic culture methods may be adapted for mass cultivation of G. intestinalis. Yields of up to 2.5-4.0 x 10^{10} trophozoites become possible with use of 19 litre glass carboys or roller bottles as culture vessels. (Farthing et al., 1982; Weider et al., 1983). It is believed that increased yields are due to an elongation of the log phase of growth rather than an increase in growth rate.

Growth of trophozoites in culture can be used as a measure of trophozoite viability. After a culture is exposed to a potentially lethal agent; the extent of culture growth over 48 hours is determined. From this cell count it is possible to extrapolate back over standard growth curves to find the original number of trophozoites killed by the test conditions used. (Hill et al., 1986).

Cultivation of the G.muris and G.agilis groups is not yet possible. Several workers have reported their efforts to culture G.muris. (*Nozaki (1956); Hasslinger (1966); Meyer, (1975) see Meyer and Radalescu 1984). Axenic culture of G.agilis has not been attempted.

1.5   Cryopreservation

Giardia trophozoites are able to survive storage at a range of temperatures (8°C to -70°C) in the presence of glycerol or dimethyl sulphoxide (DMSO).   (Bemrick, 1961; Meyer and Chadd, 1967; Warhurst and Wright, 1979).   Controlled cooling of trophozoites in 7.5% DMSO solution; and storage in liquid nitrogen until trophozoites are rapidly thawed at 37°C; gives optimal recovery of Giardia cultures after cryopreservation.   (Phillips et al., 1982, 1984).

1.6   Physiology of Growth

G. intestinalis is cultured axenically in complex, undefined media. (Kasprzak and Majewska, 1983).   Several components of these media specifically effect in vitro growth of Giardia cultures.

Ecalle (1968) and Roux and Ecalle (1968) examined the effect of pancreatic enzymes and whole pancreatic juices on the in vitro growth response of Giardia.   Without rabbit pancreatic juice, the generation times recorded in experimental cultures were 34 and 39 hours.   In the presence of 2% pancreatic juice, generation times of 31 and 39 hours were recorded.   (See Meyer and Radalescu, 1979, 1984).   Frequent replacement of media reduces the latent period of Giardia cultures ie after addition of fresh media to cultures, the exponential phase of growth appeared more rapidly (Gayrell and Ecalle (1972), see Meyer and Radalescu, 1979).

Iyer and Gaitonde (1975 a,b; 1976) (see Meyer and Radalescu, 1979) determined the influence of amino acids, pH, incubation temperature and rice starch on growth of Giardia cultures in Pavlov's medium. Histidine-HCl was found to be the only amino acid to promote culture growth.   When added to Pavlov's medium, rice starch markedly reduced the generation times of cultures.   Generation times of 5.4 and 7.8 hours after 24 and 48 hours respectively were noted.

The reducing agent L-cysteine is specifically required for growth of Giardia cultures in TY1-S-33.   In TPS-1, this reducing agent requirement is less specific.   TPS-1 which includes other sulphydryl

components, or a reduced level of L-cysteine, is able to support growth of Giardia. Media containing L-cysteine or L-Ascorbic acid alone are unable to support culture growth, but if these two components are combined, resulstant trophozoite yields may be up to 30-60% of those observed when media includes L-cysteine. (Gillin and Diamond, 1981a). L-cysteine is also specifically required for attachment of Giardia trophozoites even under an N_2 atmosphere, indicating a complex role for this thiol reducing agent. In the presence of L-cysteine, trophozoite attachment is most rapid over the first two hours of culture establishment. The numbers of attached trophozoites decreases upon exposure of cultures to media without L-cysteine. (Gillin and Diamond, 1981b).

Giardia trophozoites are oxygen sensitive. Using clonal growth of Giardia in agar to assess trophozoite viability; the protection afforded by L-cysteine against the lethal effects of oxygen has been described. (Gillin and Diamond, 1981b). Under increased oxygen tensions, L-cysteine delayed the onset of exponential killing of trophozoites. In medium without L-cysteine, decreasing oxygen tension prolonged trophozoite survival. Thiol reducing agents therefore, are required for both attachment and survival of Giardia cultures in vitro.

Trophozoite attachment in culture reaches maximum levels in media at a pH of 6.85-7.00 and an ionic strength of 200-300 mosmol/kg, NaCl being better tolerated by trophozoites than KCl. The Cohn III fraction of crude bovine serum stimulates attachment and survival of Giardia. Trophozoite attachment is temperature-dependant, and decreases at reduced temperatures. No trophozoite attachment occurs at temperatures below 12°C. (Gillin and Reiner, 1982). Human mucus from the duodenum and ileum promotes trophozoite attachment at a concentration of 100 μg/ml. It is thought that this enhancement of attachment is due to a low-density, protein-rich fraction within the mucus. (Zenian, 1985).

1.7   Trophozoite Metabolism

With successful routine axenic culture of G. intestinalis; studies of trophozoite metabolism become feasible.

Respiration of G. intestinalis is based on the incomplete oxidation of substrates such as glucose and malate to produce acetate, ethanol and CO_2.   (Lindmark, 1980; Weinbach et al., 1980; Jarrol et al., 1981).   Lindmark (1980), proposed a map of carbohydrate metabolism in G. intestinalis based on the following findings.

(i)   Trophozoites exhibited a high rate of endogenous respiration; oxygen being consumed at a rate of 93 ± 10 nmoles/min/mg protein at 37°C.   These findings were in direct contradiction to the long accepted idea of anaerobiasis in G. intestinalis.

(ii) This respiration was stimulated by glucose but not by other Krebs cycle intermediates.

(iii) Inhibition of respiration by an acetylating agent, iodoacetamide, suggested the presence of a functional glycolytic cycle.

(iv) Metabolic inhibitors cyanide, dinitrophenol and rotenone had no effect on respiration, inferring the absence of a Krebs cycle and cytochrome-based oxidative phosphorylation.

(v)   The importance of flavoproteins in electron transport was demonstrated by the inhibition of respiration due to atabrin. Quinacrine-hydrochloride (atabrin) is a flavoprotein antagonist.   (Hellerman et al., 1946).

(vi) Production of organic end products – acetate and ethanol; and CO_2, occurs under both aerobic and anaerobic conditions.

(viii) After comprehensive assays of enzyme activities, it was determined that two enzymes of glycolysis could be used to account for the observed formation of organic end products.

Some enzyme systems in _Giardia_, were found to be similar to those in _Entamoeba_ and _Trichomonad_ species. (Respiratory systems in both these genera were found to be useful analogues for the respiratory system in _G. intestinalis_ by many workers). (Lindmark, 1980; Weinbach _et al._, 1980; Jarrol _et al._, 1981).

Figure 1:   Carbohydrate metabolism in G. intestinalis.
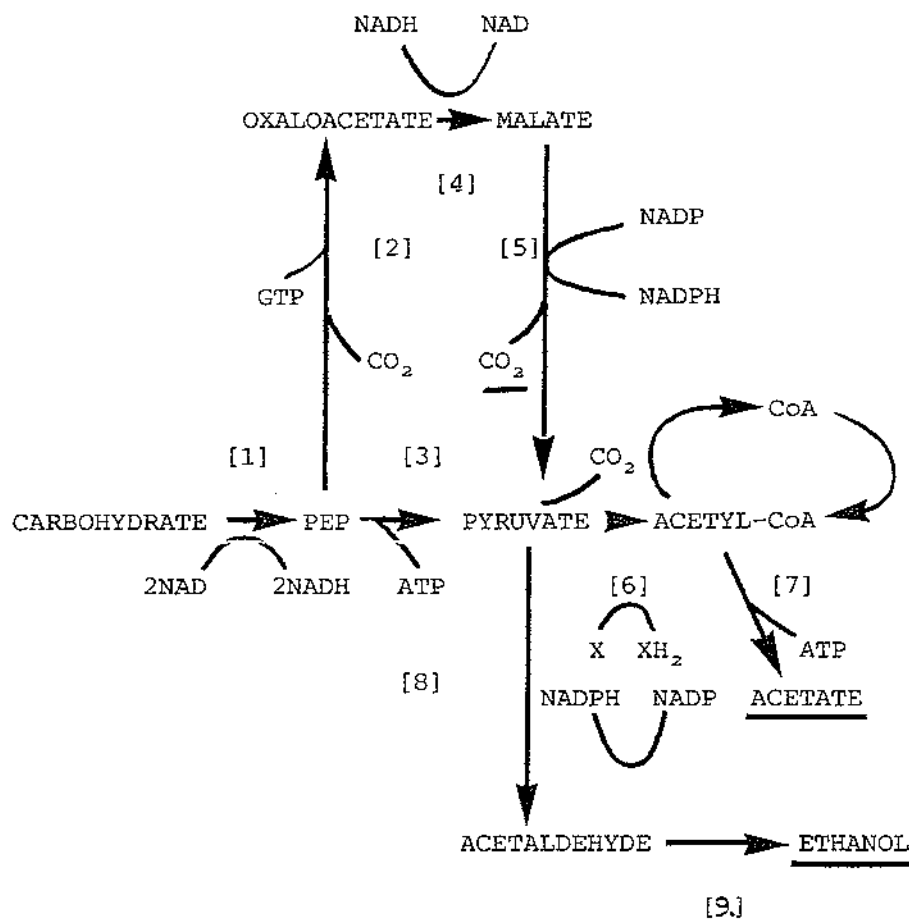
[1] Glycolytic enzymes

[2] PEP Carboxykinase (GDP)

[3] Pyruvate kinase (ADP)

[4] Malate dehydrogenase (NAD)

[5] Malate dehydrogenase (carboxylating) (NADP)

[6] Pyruvate synthetase (ADP)

[7] Acetyl CoA synthetase (ADP)

[8] Alcohol dehydrogenase (NADP)

[9] NADPH Oxidoreductase

Lindmark's findings were confirmed by Weinbach et al., (1980); who demonstrated that trophozoites consume oxygen at a high rate, irrespective of oxygen concentrations until a zero oxygen level is approached. He found that glucose; as well as malate and ethanol stimulate respiration at least two-fold, with Kreb's cycle intermediates having no effect on oxygen consumption. Suppression of respiration by a number of metabolic inhibitors was also confirmed. From observing the stimulation of respiration after the addition of NAD(P)H to the sedimented particulate fraction of disrupted trophozoites; Weinbach et al., concluded that G. intestinalis has an active particulate DT-Diaphorase. (An enzyme or enzymes which catalyse the oxidation of NADH and NADPH at equal rates). (Ernster, 1967).

Flavins in Giardia are acid extractable. (Weinbach et al., 1980). Weinbach stated that these free flavins were further implication of a lack of mitochondria and tricarboxylic acid enzymes, since where a Kreb's cycle is present, flavins are covalently bound in flavonucleotide complexes (FAD), with succinate dehydrogenase. During further study of potential electron carriers in Giardia; iron not bound to heme proteins was detected and sulphides were found to be acid labile. Weinbach described these findings as 'prima-facie' evidence for the presence of Fe-S centres. Subsequent demonstration of EPR-spectra characteristic of iron-sulfur proteins confirmed this.

Generally; energy production in G. intestinalis is by substrate-level phosphorylation, and a flavin, iron-sulfur protein mediated electron transport chain. Cytochrome mediated oxidative phosphorylation and a functional Kreb's cycle are absent. (Lindmark, 1980).

Trophozoites incorporate cholesterol and fatty acids from growth medium. They do not utilise lipid precursors. (Jarrol et al., 1981). G. intestinalis is suggested to be incapable of de novo synthesis of lipids, though this synthesis could be repressed due to high lipid levels in media.

Giardia trophozoites readily incorporate pyrimidines and pyrimidine nucleosides into nucleic acids. (Lindmark and Jarrol, 1982). Enzymes of the pyrimidine salvage pathway; (thymidine phosphorylase, uridine phosphorylase, thymidine kinase and uridine kinase) have been described. These enzymes are non-sedimentable, and so are not associated with subcellular organelles. Activity of de novo pyrimidine synthesis enzymes; (carbamoyl-phosphate synthase, aspartate transcarbamoylase, dihydroorotase and *dihydroorotase dehydrogenase); has not been detected. (Lindmark and Jarrol, 1982).

The unique metabolism of G. intestinalis may be of medical interest. Preliminary characterisation of the activity of the Giardia nucleoside phosphotransferase enzyme, has determined that the enzyme has substrate and phosphate-donor specificities which differ from the mammalian phosphotransferase. This enzyme in Giardia has been suggested as a site for chemotherapeutic attack. (Berens and Marr, (1986).