Giardia intestinalis :

Aerobic Metabolism and Physiology of *in vitro* Growth.

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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^{o}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^{o}C (20^{o}C and 30^{o}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 G. intestinalis 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 eliptically 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) G. muri group: - 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 et al., 1986).

1.1.3 Excystation and Encystment

The process of excystation has only been seen and described this century. Several early reports described in vivo excystation occurring in faeces and intestines of hosts (Hegner (1925a, 1927c), see Filice, 1952). A partial in vitro excystation of Giardia 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 et al., 1979; Feely et al., 1984; Buchel et al., 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; in vitro excystation is now routinely possible using cysts from a variety of animal sources. (Bingham et al., 1979; Rice and Schaefer, 1981a; Schaefer et

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

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 TYI-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.

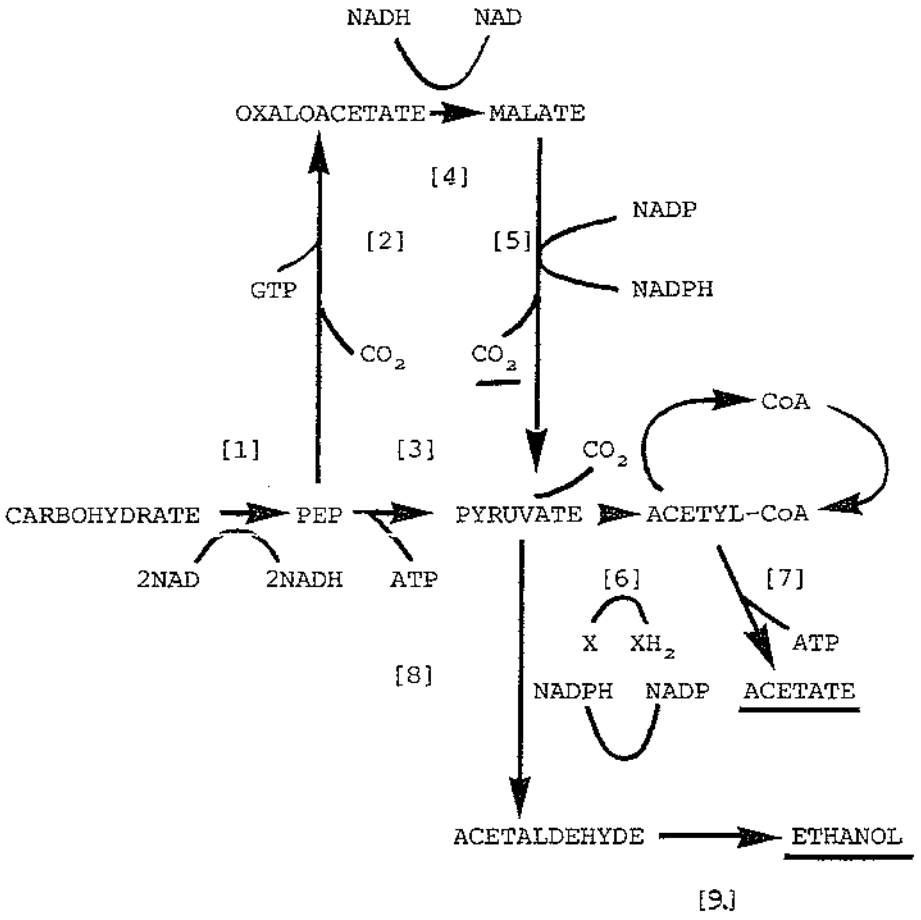
(v) The importance of flavoproteins in electron transport was demonstrated by the inhibition of respiration due to atabrin. Quinacrine-hydrochloride (atabrin) is a flavoprotein antagonist. (Hellerman et al., 1946).

(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).

CHAPTER TWO : MATERIALS

2.1 Axenic Cultures

A. [a]Bris/83/HEPU/106: Source: Duodenal biopsy of an infected symptomatic child. (Courtesy of Dr P F L Boreham, Queensland Institute of Medical Research).

B. Hast/87/MUGU/68: Source: Animal model for giardiasis, ie, trophozoites obtained by duodenal biopsy after *in vivo* excystation with suckling mice.

a. Nomenclature of strains (Boreham *et al.*, 1984): 83/87 - Year of recovery
HEPU/MUGU - Laboratory where strain was isolated and cultivated.
106/68 - Laboratory sample number.

2.2 Axenic Growth Media

2.2.1 TY1-S-33

The following components were combined with 500 ml Milli-Q water. (Many components of this medium are complex, and may differ from batch to batch in their ability to support growth of *Giardia* (Kasprzak + Majewska, 1985). Where this is the case, the source of the component has been quoted).

Trypticase Soy Broth BBL (11768) and Oxoid (M129)	20.0 g
Yeast Extract (Difco 0127-01)	10.0 g
Glucose	10.0 g

NaCl	2.0 g
K_2HPO_4	1.0 g
KH_2PO_4	0.6 g
L-cysteine Monohydrochloride (Sigma C-7880)	1.5 g
Ferric Ammonium Citrate (Brown Pearls)	0.023 g
L-Ascorbic acid	0.2 g
[a]NCTC 135 (Gibco 440-1100) or NCTC 109 (Difco 5927-23)	100 ml
Bile Bacteriological (Sigma B 8381)	0.8 g
Benzylpenicillin	0.06 g
Gentamicin sulphate	0.05 g
Vancomycin	0.02 g
[b]Bovine Serum	100 ml

Milli-Q water was added to bring the total volume to 1 l.

The media was clarified by positive-pressure membrane filtration through cellulose nitrate papers (0.45 µm pore size).

1.0 mol l^{-1} NaOH was used to adjust the pH of the media to 7.0-7.2.

After filter-sterilization by positive pressure through 0.2 µm pore membrane filters, the medium was stored at 4°C for a maximum of 12 days.

a **Preparation of NCTC broth:**

NCTC 1 35 or NCTC 109	9.7 g
Milli-Q water up to	1 l.

The above constituents were combined and sterilized by membrane filtration.

NCTC broth was stored at 4°C.

b **Bovine serum was obtained through two sources:**

I A membrane filtered product was acquired through Gibco (NZ).

II Serum was prepared in the laboratory by centrifuging of blood obtained from the Waitaki Freezing Works in Fielding.

2.2.2 Agarose Stock Solution

Agarose III (Sigma) was added to TY1-S-33 medium base to a final concentration of 2.5% w/v.

TY1-S-33 medium base: (Constituent sources as in 2.2.1).

Trypticase Soy Broth	2.0 g
Yeast Extract	1.0 g
Glucose	1.0 g
NaCl	0.2 g
K_2HPO_4	0.1 g
K_2HPO_4	0.06 g
l-Cysteine monohydrochloride	0.15 g
Ferric Ammonium Citrate	0.0023 g
Bile Bacteriological	0.08 g
Milli-Q water to	100 ml

The stock solution was autoclaved at 15 lb/sq in for 15 mins and kept at 65°C until used.

2.3 Miscellaneous Media

2.3.1 Hanks Balanced Salt Solution (HBSS)

Stock Solution A:	I	NaCl	16.0 g
		KCl	0.8 g
		$MgSO_2.7H_2O$	0.2 g
		$MgCl_2.6H_2O$	0.2 g
		Milli-Q water to	80 ml

II	$CaCl_2$	0.28 g
	Milli-Q water to	10 ml

Solutions I and II were combined slowly. Milli-Q water was used to bring the total volume of stock Solution A to 100 ml.

This solution was stored at 4°C with 0.2 ml chloroform.

Stock Solution B:	$NaHPO_4.12H_2O$	0.304 g
	KH_2PO_4	0.12 g
	Glucose	2.0 g
	Milli-Q water to	80 ml

10 ml 0.4% phenol red in NaOH was added to this solution. Milli-Q water was used to bring the total volume of Stock Solution B to 100 ml.

This solution was stored at 4°C with 0.2 ml chloroform.

For use in axenic culture and experimental work, HBSS was prepared as follows:

Stock Solution A	10 ml
Stock Solution B	10 ml
Milli-Q water to	80 ml

HBSS was sterilized by positive-pressure membrane filtration through cellulose nitrate filters (0.2 μm pore size); and stored at 4°C.

2.3.2 Phosphate Buffered Saline

NaCl	9.5 g
$Na_2HPO_4.12H_2O$	13.5 g
$NaH_2PO_4.2H_2O$	0.32 g
Milli-Q water to	1000 ml

The above components were combined, autoclaved at 15 lb/sq in for 15 minutes, and stored at 4°C.

CHAPTER THREE : METHODS

3.1 Enumeration

3.1.1 'Free' or 'Non-Adherent' Trophozoites

'Free' trophozoites are those suspended in growth medium at 37°C at the time of enumeration. This usually represents approximately 10% of the total trophozoite population in culture. To count these trophozoites, the following protocol was followed.

i. A small aliquot of growth medium was removed from a culture tube at 37°C.
ii. The number of trophozoites in this aliquot was determined using a hemocytometer under phase contrast.
iii. After duplicate counts had been made, the number of free or non-attached trophozoites in the culture tube was determined.

3.1.2 'Attached' or 'Adherent' Trophozoites

i. Expired medium in the culture tube was discarded.
ii. The empty culture tube was then filled with chilled HBSS and placed in an ice-water bath for 10 minutes.
iii. Once all trophozoites had detached from the culture tube surface, a small aliquot of this trophozoite suspension was removed.
iv. The number of trophozoites in this aliquot was determined by following the protocol described for enumeration of non-attached trophozoites.

3.1.3 Determination of the Growth Rate of Experimental Cultures

i. Duplicate tubes containing cultures in early log phase (24-48 hours) were placed in an ice-water bath for 10 minutes.

   Since original growth medium was not discarded, the resultant trophozoite suspension consisted of both 'free'

and formerly adherent trophozoite populations.

ii. A small aliquot of this trophozoite suspension was removed from each culture tube.

iii. Following the protocol described earlier; the total number of trophozoites in each culture tube was determined.

iv. This enumeration process was repeated during the late log phase of culture growth (72-96 hours). To determine the growth rate of experimental cultures over this period of logarithmic growth, the following formula was used:

$$\text{Growth rate} = \frac{1}{a} = \frac{\log n_2 - \log n_1}{\log 2.\ (t_2 - t_1)} = \text{generations/hour}$$

Where t_1 = age of culture at time of first trophozoite count.
t_2 = age of culture at time of second trophozoite count.
n_1 = trophozoite population at t_1.
n_2 = trophozoite population at t_2.

Unless otherwise stated, all experimental cultures were initiated using adherent trophozoites obtained according to the protocol described earlier. (See 3.1.2).

3.2 In vitro Growth Requirements

3.2.1 Determining the Effect of pH on the Growth of Experimental Cultures

i. A series of 150 ml aliquots of TYI-S-33 were adjusted with 1.0 M HCl and 1.0 M NaOH to produce the following range of pH levels:

6.0, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, 7.0.

ii. Each aliquot of experimental TYI-S-33 (Gillin and Diamond, 1981) was dispensed into fourteen 13 x 100 mm culture tubes.

iii. Cultures in these tubes were initiated with 1.0×10^5-5.0×10^5 trophozoites, and incubated at $37^{\circ}C$.

iv. Duplicate tubes were removed daily and counts were made of the free, adherent, and total cell populations at each pH tested. (See 3.1).

3.2.2 Determining the Effect of Incubation Temperature on the Growth of Experimental Cultures

i. Parallel series of cultures containing 1.0×10^4 - 5.0×10^4 trophozoites/ml were incubated at the following temperatures for seven days:
25^oC, 30^oC, 37^oC, 40^oC, 45^oC.

ii. Duplicate culture tubes at each temperature were removed daily.

iii. Enumeration of free, adherent, and total trophozoite populations in the experimental cultures was carried out following the protocol described earlier. (See 3.1).

3.3 Clonal Growth in Agarose as an Assay of Trophozoite Viability (Gillin and Diamond, 1980)

3.3.1 Preparation of Trophozoite Suspension

i. A chilled suspension of adherent trophozoites in Hank's Balanced Salt Solution (HBSS) was prepared according to the protocol in 3.1.2.

ii. After a hemocytometer had been used to determine the concentration of the trophozoite suspensions HBSS was used to dilute the cell suspension to a final concentration of 6.0×10^3 cells/ml.

3.3.2 Establishment of Clonal Growth in Agarose

i. A concentrated stock solution (2.5% w/v) of agarose in TY1-S-33 medium base was prepared; sterilized by autoclaving; and kept at 65^oC.

ii. 8 ml aliquots of complete, sterile TY1-S-33 were dispensed to culture tubes (13 x 100 mm) which were warmed to 42^oC.

iii. Appropriate volumes of agarose stock solution were added to warmed 8 ml aliquots of TY1-S-33 to the following range of final agarose concentrations (% w/v).

0.18, 0.22, 0.25, 0.29

The contents of the tubes were mixed by inversion, and

kept at 42°C for 10 minutes.

iv. Samples of the diluted cell suspension (3.3.1) were added to the agarose solutions at a range of 100-1000 trophozoites/culture tube. Trophozoites were dispenred by repeated inversion of culture tubes.
Small inocula allowed for ease in counting of discrete colonies after incubation.

v. After inoculation, culture tubes were either cooled in an ice-water bath for 10 minutes, or left at room temperature for 1 hour, before being transferred to 37°C incubation.

vi. After 7-8 days incubation at 37°C, discrete colonies dispersed in the semi-solid agarose matrix were visible to the naked eye and were counted.
Comparing colony growth with the original number of trophozoites in each culture tube provided a quantitative estimate of culture viability under various test conditions.

$$\text{CFE (\% colony forming efficiency)} = \frac{\text{no of colonies} \times 100}{\text{no of cells inoculated}}$$

3.4 The Effect of L-Cysteine Concentration and Redox Potential on Trophozoite Attachment and Culture Growth in TY1-S-33

3.4.1 Reducing Conditions and Culture Growth

i. TY1-S-33 growth medium lacking L-cysteine was prepared and stored at 4°C.

ii. To six aliquots of this medium, appropriate amounts of L-cysteine were added, giving the following concentrations of L-cysteine in TY1-S-33 (% w/v).

0.00, 0.05, 0.1, 0.15, 0.2, 0.25

Each aliquot was then filter-sterilized according to the protocol for preparation of TY1-S-33.

iii. A model 96-78.00 platinum Redox electrode with an expandable ion Analyzer EA 920 (Orion Research) was used to measure the oxidation-reduction potentials of these six experimental media at pH 7.15, 37°C.

The O-R potentials obtained were as follows:

Table I: Reducing Conditions Produced in TY1-S-33 Growth Medium

L-Cysteine Conc. (% w/v)	O-R Potential (mV)
0.00	-114.7
0.05	-191.5
0.10	-243.6
0.15	-272.4
0.20	-289.8
0.25	-302.1

iv. Each aliquot of experimental TY1-S-33 was dispensed into a series of 13 x 100 mm culture tubes.

v. Half the culture tubes were inoculated with 6.50 x 10^4-1.5 x 10^5 trophozoites and incubated at 37°C.

vi. Duplicate culture tubes from each of the L-cysteine concentrations tested were removed daily.

vii. Enumeration of free, adherent and total trophozoite populations in the experimental cultures was carried out following the protocol described earlier. (See 3.1).

3.4.2 Reducing Conditions and Trophozoite Attachment

i. The remaining culture tubes containing experimental media were inoculated with 1.5-2.6 x 10^5 trophozoites and incubated at 37°C.

ii. The numbers of free and adherent trophozoites in the culture tubes were determined at 30 minute intervals during the four hours immediately following culture initiation.

iii. Enumeration was carried out according to the protocol described earlier. (See 3.1).

3.5 Sensitivity of Trophozoites to Elevated Dissolved Oxygen (D.O) levels in TY1-S-33

3.5.1 Preparation of Trophozoite Suspensions

i. Adherent trophozoites from five culture tubes were harvested according to the protocol in 3.1.2. to provide a final total trophozoite yield of 1.0-5.0 x 10^7 cells.

ii. Cell suspensions were centrifuged at 1200 x g for 10 minutes; the supernatant was discarded, and the trophozoites were resuspended in 1 ml chilled HBSS.

iii. The concentration of each cell suspension was determined using a hemocytometer under phase contrast.
These trophozoites were maintained at 4°C to prevent reattachment of cells to culture tube surfaces.

3.5.2 Exposure of Trophozoites to Elevated D.O. Levels in TYI-S-33.

i. A Corning 1000 ml 'Quickfit' Culture Vessel was completely filled with TYI-S-33 growth medium and adjusted to appropriate temperatures in either a heated water bath for temperatures 30°C and above; or an L.K.G. Bromma 2209 Multitemp water bath for temperature below 30°C.

ii. Pure oxygen was bubbled through the temperature-adjusted medium until the desired D.O. level was reached.
A model 97-08 Oxygen Electrode with an expandable ion analyzer EA 920 (Orion Research) was used to measure dissolved oxygen (ppm) at test temperatures.

iii. 10-16 15 ml sample vials were immersed in the contents of the culture vessel.

iv. The trophozoite suspension prepared in 3.5.1 was added to the culture vessel.

v. Oxygen was constantly bubbled through the growth medium to maintain an elevated oxygen tension. Oxygen flow was controlled with a Matheson 600 Series 150 mm Flowmeter unit.

3.5.3 Enumeration of Viable Trophozoites

i. At hourly intervals a sample vial was removed from the

culture vessel.

ii. Growth medium containing 'free' trophozoites was transferred to 13 x 100 mm culture tubes and chilled.

iii. The sample vial was then filled with chilled HBSS and placed in an ice-water bath for 10 minutes to allow for trophozoite detachment. The resultant trophozoite suspension was transferred to 12 x 100 m culture tubes.

iv. All culture tubes were centrifuged at 1200 x g for 10 minutes; the supernatant was discarded, and the trophozoites were resuspended in 1 ml chilled HBSS.

v. Enumeration of viable 'free', adherent and total trophozoite populations was carried out according to the protocol in 3.1.

Trophozoites were deemed non-viable if gross morphology was distorted and flagellar movement had ceased.

From enumeration of trophozoite numbers in sample vials, the trophozoite population of the 1000 ml culture vessel was estimated.

3.6 Oxygen Consumption by G. intestinalis

3.6.1 Changes in the D.O. Level of Sterile PBS at 37°C

i. Sterile PBS solution was warmed to 37°C in a water bath, and transferred to a 250 ml Kimax flask, filling it completely.

ii. A model 97-08 Oxygen Electrode (Orion Research) was inserted in the neck of the flask and parafilm was used to provide an airtight seal,.

iii. While the contents of the flask were stirred constantly; the changes in the D.O. level of the PBS were measured at 30 minute intervals using an Expandable ion Analyzer EA 920 (Orion Research).

3.6.2 Oxygen Consumption by Trophozoites in PBS

The procedure as in 3.6.1 was followed, with the addition of 6.8×10^7 - 2.6×10^8 trophozoites[a] prior to sealing of the

flask.

3.6.3 Changes in the D.O. Level of Sterile Quinacrine dihydrochloride (1000 µg/ml) in PBS

The procedure as in 3.6.1 was followed, with the addition of Quinacrine dihydrochloride[b] to PBS at a final concentration of 1000 µg/ml prior to sealing of the flask.

3.6.4 The Effect of Quinacrine dihydrochloride on Oxygen Consumption by Trophozoites in PBS

i. Quinacrine dihydrochloride was added to aliquots of PBS to the following concentrations: (µg/ml): 1.0, 100, 250, 500, 1000.
ii. Each of these aliquots was transferred to 250 ml Kimax flasks as in 3.6.1.
iii. Each flask in turn was inoculated with 2.3 - 2.7 x 10^8 trophozoites and sealed with an Oxygen Electrode which was used to measure changes in D.O. level as in 3.6.1.

a Preparation of 'high-yield' trophozoite suspensions:
i. 150 ml tissue culture bottles completely filled with TYI-S-33 growth medium were inoculated with 1.0-5.0 x 10^5 trophozoites.
ii. Cultures were grown to log phase (72-96 hours).
iii. Non attached trophozoites were discarded and attached trophozoites suspended in chilled HBSS as in 3.1.2.
iv. The trophozoite suspensions were transferred to 13 x 100 mm culture tubes and centrifuged at 1200 g for 10 minutes.
v. After the supernatant was discarded; 1 ml of chilled HBSS was used to resuspend the trophozoites.
vi. The final concentration of the trophozoite suspension was determined using a hemocytometer as in 3.1.1.

b Preparation of Quinacrine dihydrochloride Solution. (See Appendices).

3.6.5 Viability of Trophozoites After Exposure to Quinacrine dihydrochloride

3.6.5(a) Exposure to Quinacrine dihydrochloride in Semi-Solid Agarose Medium

i. Appropriate volumes of 10 μg/ml Quinacrine dihydrochloride were added to TY1-S-33 growth medium in 13 x 100 mm culture tubes to produce the following range of concentrations (μg/ml):
0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2.

ii. All tubes were warmed to 42°C in a water bath.

iii. Agarose stock solution was added to these culture tubes to a final concentration of 0.18% w/v agarose.

iv. The protocol for the establishment of clonal growth of Giardia in agarose medium (3.3) was then followed.

3.6.5(b) Viability of Trophozoites After 5 Hours Exposure to Quinacrine dihydrochloride.

i. Appropriate volumes of 10 μg/ml Quinacrine dihydrochloride were added to PBS solution in 13 x 100 mm culture tubes to produce the following range of concentrations (μg/ml):
0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0.

ii. All culture tubes were inoculated with 1.0 - 5.0 x 10^5 trophozoites, and incubated at 37°C for 5 hours.

iii. The cultures were then chilled in an ice-water bath for 10 minutes and centrifuged at 1200 g for 10 minutes.

iv. After the supernatant had been discarded, the trophozoites were resuspended in chilled HBSS and centrifuged again at 1200 g for 5 minutes.
Trophozoites were washed using the above protocol three times.

v. Resultant trophozoite suspensions in PBS were

diluted to appropriate concentrations.

vi. Culture tubes containing TYI-S-33 growth medium and agarose stock solution to a final concentration of 0.18% w/v were warmed to 42°C.

vii. Washed trophozoite suspensions were used to inoculate these tubes at a concentration of 100-1000 trophozoites/culture tube according to the protocol described in 3.3.1.

viii. Inoculated agarose solutions were allowed to gel for 1 hour at room temperature before being incubated at 37°C for 7-8 days.

CHAPTER FOUR : RESULTS

4.1 The Effect of pH and Temperature on the Growth of Two Strains of G. intestinalis

Routine axenic cultivation of G. intestinalis has only been possible since 1976 (Meyer, 1976). Difficulties in the establishment of axenic cultures have been largely due to the fastidious nature of the organism (Kasprzak and Majewska, 1985); with the extent of culture growth being dependent on environmental conditions.

The influences of environmental factors such as temperature and pH on trophozoite attachment in culture have been demonstrated. (Gillin and Reiner, 1982). The following experiments were designed to investigate correlations between these same environmental factors, and the in vitro growth rate of established axenic cultures. In the first set of experiments, the temperature range and optimum for growth of G. intestinalis in liquid culture were determined. The growth rates of experimental cultures at test temperatures are plotted in Figs. 4-5 and Tables XI - XII.

Strain Bris/83/HEPU/106 was successfully cultivated over a very limited temperature range. Rapid cell death occurred at 25°C and 45°C. Gradual loss of culture viability was recorded at 30°C.

In contrast to Bris/83/HEPU/106; HAST/87/MUGU/68 had a greater 'temperature tolerance' with culture growth observed over the range 30-40°C, and only a slow decline in trophozoite numbers occurring at 25°C. For both strains the optimum incubation temperature was 37°C. Growth of cultures continued at 40°C but at a greatly reduced rate.

Growth of cultures in TY1-S-33 growth medium was dependent on the pH of that medium. (See Figs 6-7 and Tables XIII-XIV).

For both strains tested, growth rates increased gradually with

pH until maximum growth was obtained under optimal pH conditions; (Bris/83/HEPU/106: pH 7.0-7.5; HAST/87/MUGU/68; pH 6.75-7.25). At pH levels above these optima; increasing pH was coupled with rapidly declining culture growth rates. This effect was most marked in Hast/87/MUGU/68.

All results expressed are the mean of 3-4 determinations.

Unless otherwise stated, the following legend applies to all figures:

● = free or non-adherent trophozoites
○ = attached or adherent trophozoites
◐ = total trophozoite population.

Figure 2: Growth of G. intestinalis Strain Bris/83/HEPU/106 in TY1-S-33 Growth Medium.

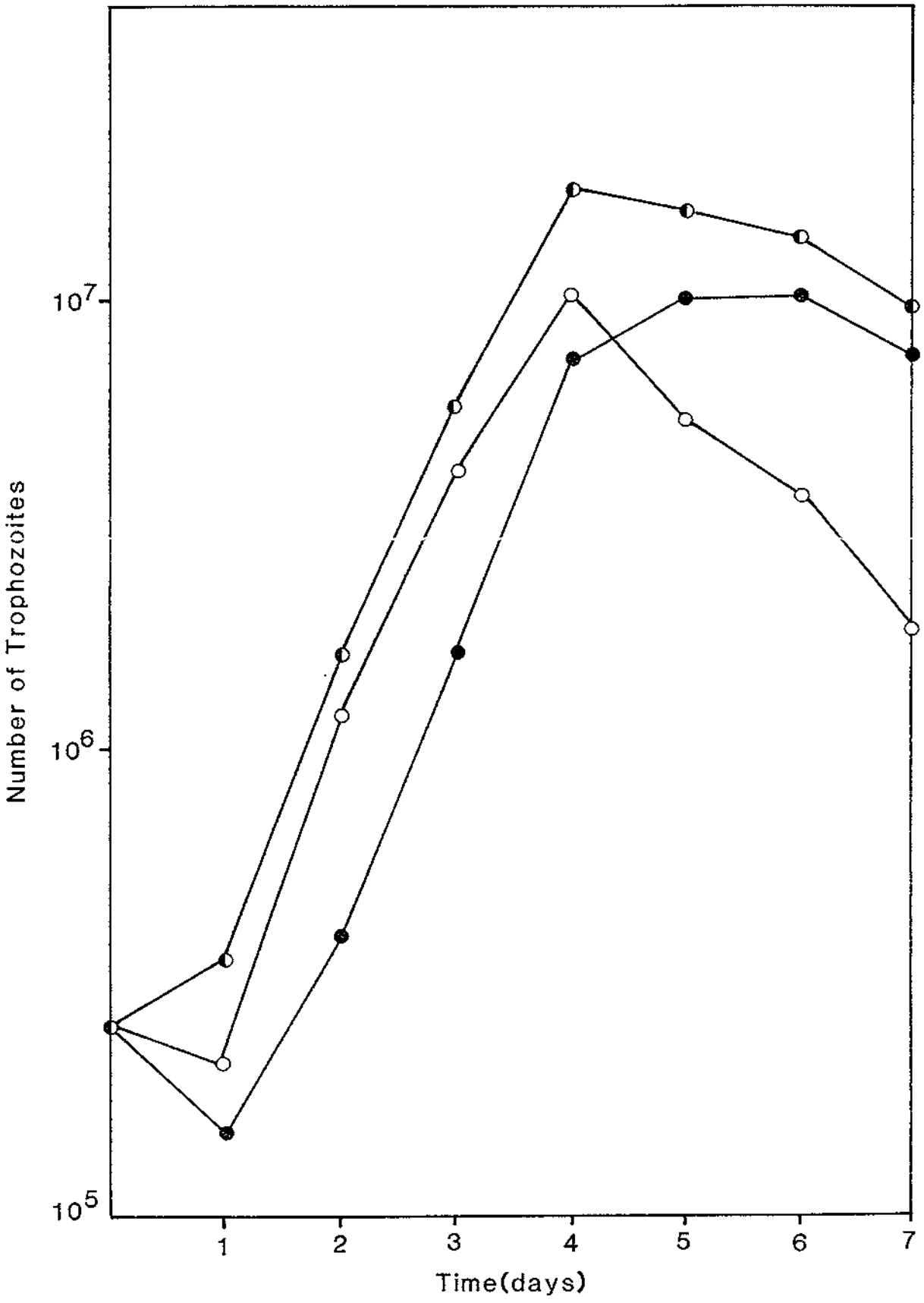


Figure 3: Growth of G. intestinalis Strain Hast/87/MUGU/68 in TY1-S-33 Growth Medium.

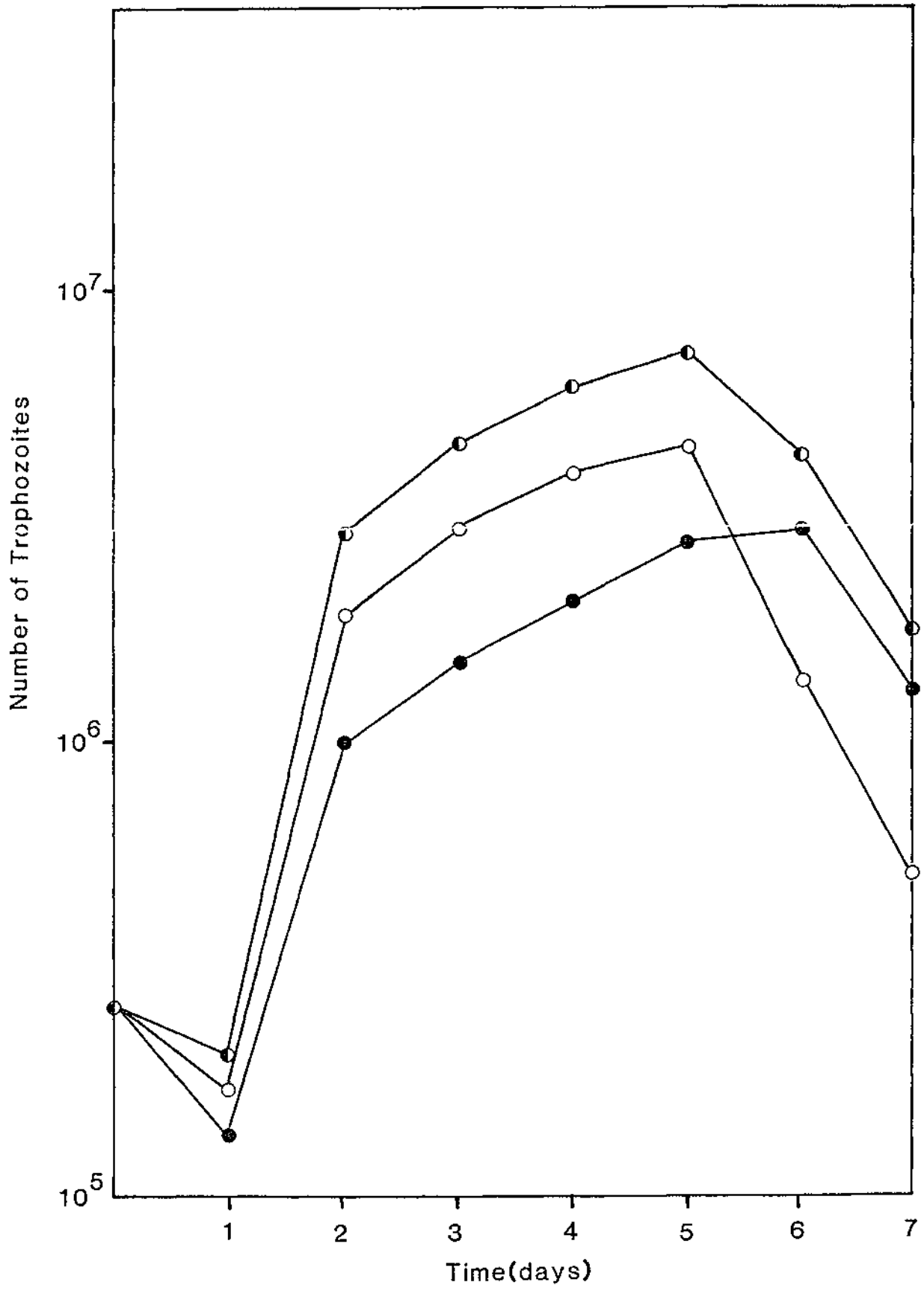


Figure 4: The Effect of Incubation Temperature on Growth of Strain Bris/83/HEPU/106.

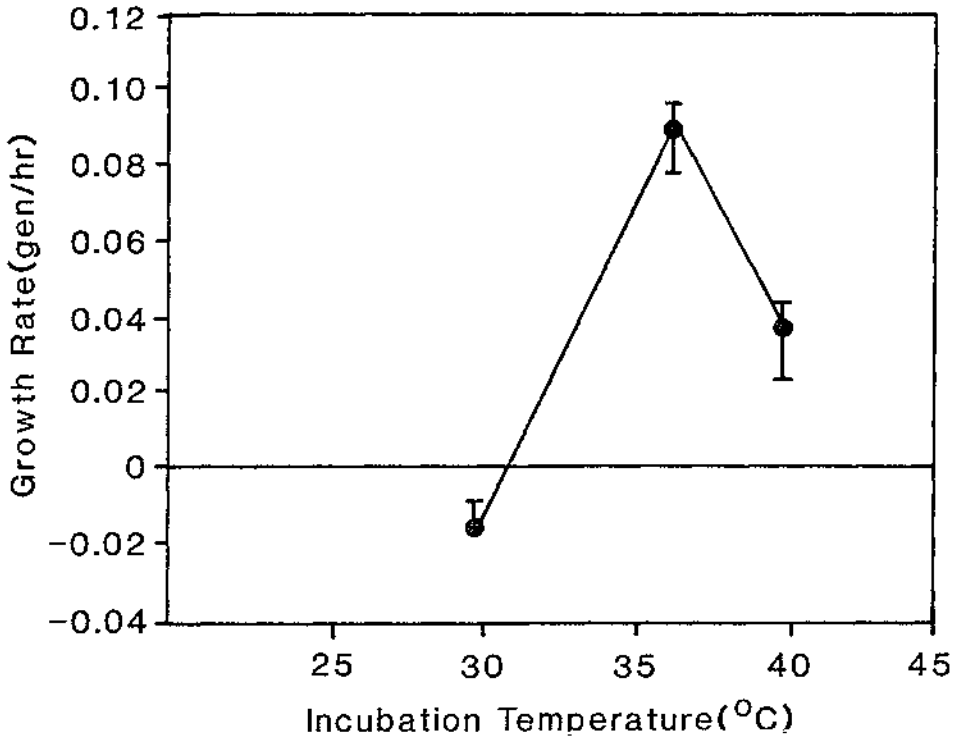


Figure 5: The Effect of Incubation Temperature on Growth of Strain Hast/87/MUGU/68.

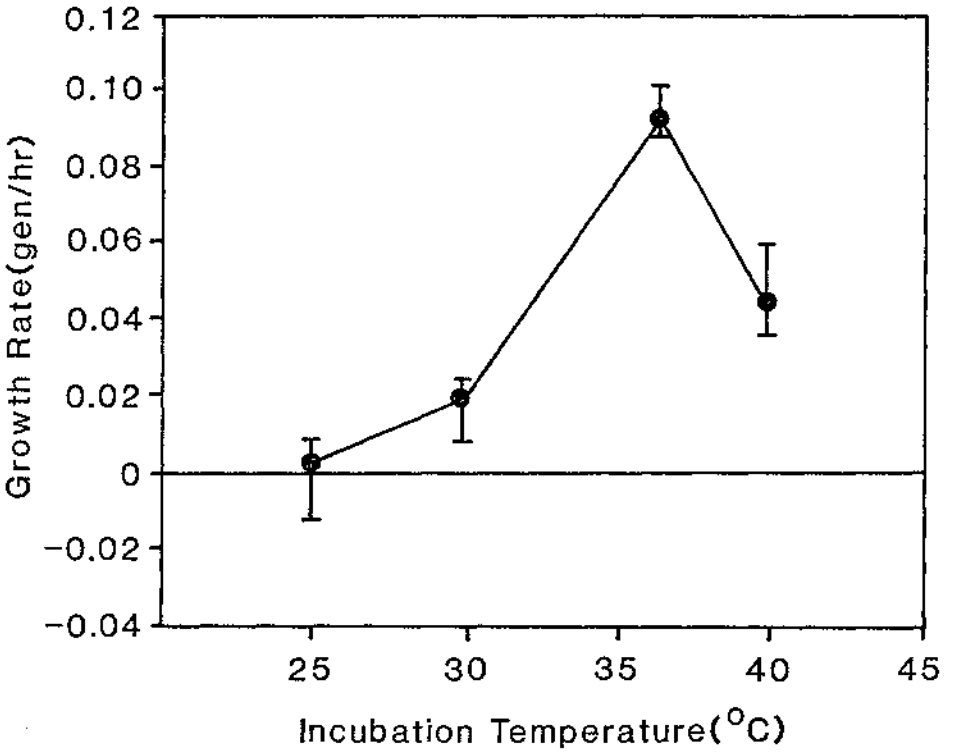


Figure 6: The Effect of pH on Growth of Strain Bris/83/HEPU/106.

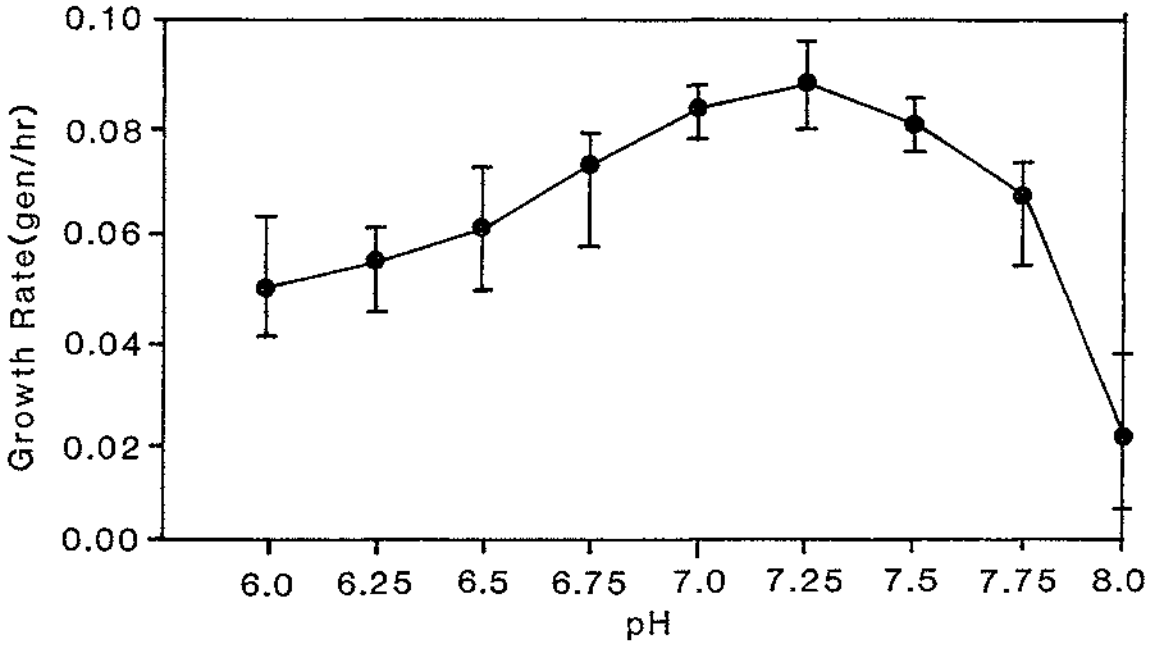
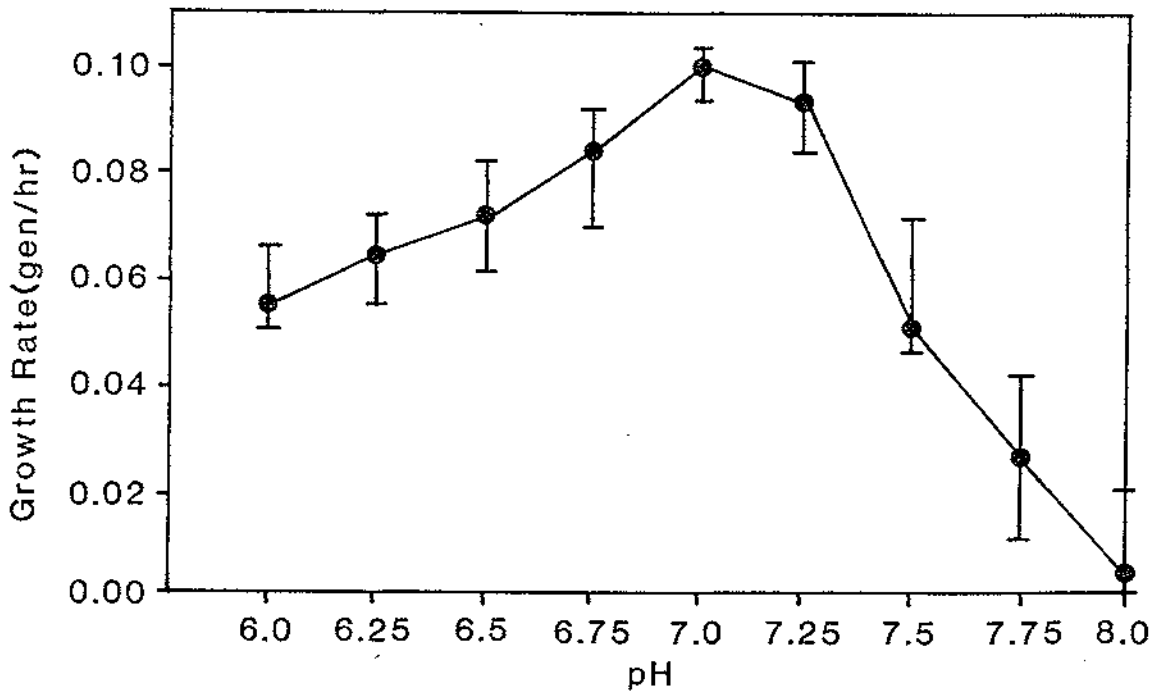


Figure 7: The Effect of pH on Growth of Strain Hast/87/MUGU/68.



4.2 Clonal Growth of G. intestinalis in Semi-solid Agarose Medium

Clonal growth of G. intestinalis trophozoites in semi-solid agarose medium was first described by Gillin and Diamond (1980). This method of assaying trophozoite viability was considered to be 'quantitative, sensitive and convenient'. Since determination of trophozoite viability under various test conditions constituted a large proportion of this study, it was felt necessary to reassess the effectiveness of this assay, and determine if theee were 'clear-cut' optimal conditions for growth of G. intestinalis in agarose medium.

Tables II - III illustrate the variability of such a method in this laboratory. Of the two strains examined; HAST/87/MUGU/68 was best adapted to growth in agarose, with colony forming efficiencies of up to 60% recorded. Both Bris/83/HEPU/106 and HAST/87/MUGU/68 grew most efficiently in 0.18% w/v final concentration agarose.

Chilling of culture tubes in an ice water bath prior to incubation at 37^{o}C (see 3.3.2); resulted in an accumulation of trophozoites at the base of the tube, as opposed to the ideal dispersion of discrete colonies throughout the agar matrix.

Due to the observed variability of colony forming efficiency (CFE), it was preferable not to employ clonal growth in agar as a measure of trophozoite survival under elevated oxygen tensions in liquid media (see 4.4).

Table II: Clonal Growth of Strain Bris/83/HEPU/106 in Semi-Solid Agarose Medium.

i. Culture tubes allowed to gel at room temperature for 1 hour before incubation at 37°C.

Agarose Concentration (Final % w/v)	Inoculum (cells/tube)	No of Colonies	CFE (%)
0.18	100	21	21
	300	57	19
	600	138	23
	1000	109	11
0.22	100	33	33
	300	-	-
	600	72	12
	1000	-[a]	-
0.25	100	19	19
	300	40	13
	600	-[a]	-
	1000	-[a]	-

ii. Culture tubes allowed to gel in an ice-water bath for 10 minutes before incubation at 37°C.

Agarose Concentration (Final % w/v)	Inoculum (cells/tube)	No. of Colonies	CFE(%)
0.18	100	32	32
	300	86	29
	600	73	12
	1000	78	8
0.22	100	-[c]	-
	300	60	2
	600	70	12
	1000	-[a, c]	-
0.25	100	20	20
	300	44	15
	600	52	9
	1000	-	-

Table III: Clonal Growth of Strain Hast/87/MUGU/68 in Semi-Solid Agarose Medium.

i. Culture tubes allowed to gel at room temperature for 1 hour before incubation at 37°C.

Agarose Concentration (Final % w/v)	Inoculum (cells/tubes)	No of Colonies	CFE(%)
0.18	100	40	40
	300	129	43
	600	-a	-
	1000	-a	-
0.22	100	60	60
	300	-a	-
	600	-a	-
	1000	-a	-
0.25	100	41	41
	300	161	53
	600	-b	-
	1000	-b	-

ii. Culture tubes allowed to gel in an ice-water bath for 10 minutes before incubation at 37°C.

Agarose Concentration (Final % w/v)	Inoculum (cells/tubes)	No of Colonies	CFE(%)
0.18	100	-c	-
	300	82	27
	600	57	9.5
	1000	-a, c	-
0.22	100	15	15
	300	112	37
	600	-a, c	-
	1000	-a, c	-
0.25	100	23	23
	300	70	23.5
	600	-b	-
	1000	-b	-

a Colonies too numerous to count
b Colonies too numerous and too small to be counted with naked eye
c Most trophozoites accumulated at the base of the culture tube.

CFE are tabulated from the mean of 2-4 determinations.

Plate 1. Macroscopic Appearance of Bris/83/HEPU/106 Colonies in Agarose Medium.

A. 0.22% w/v Agarose
Inoculum = 600 cells/tube

B. 0.18% w/v Agarose
Inoculum = 900 cells/tube

C. 0.18% w/v Agarose
Inoculum = 600 cell/tube

Note colonies appear less diffuse in 0.22% w/v agarose medium.

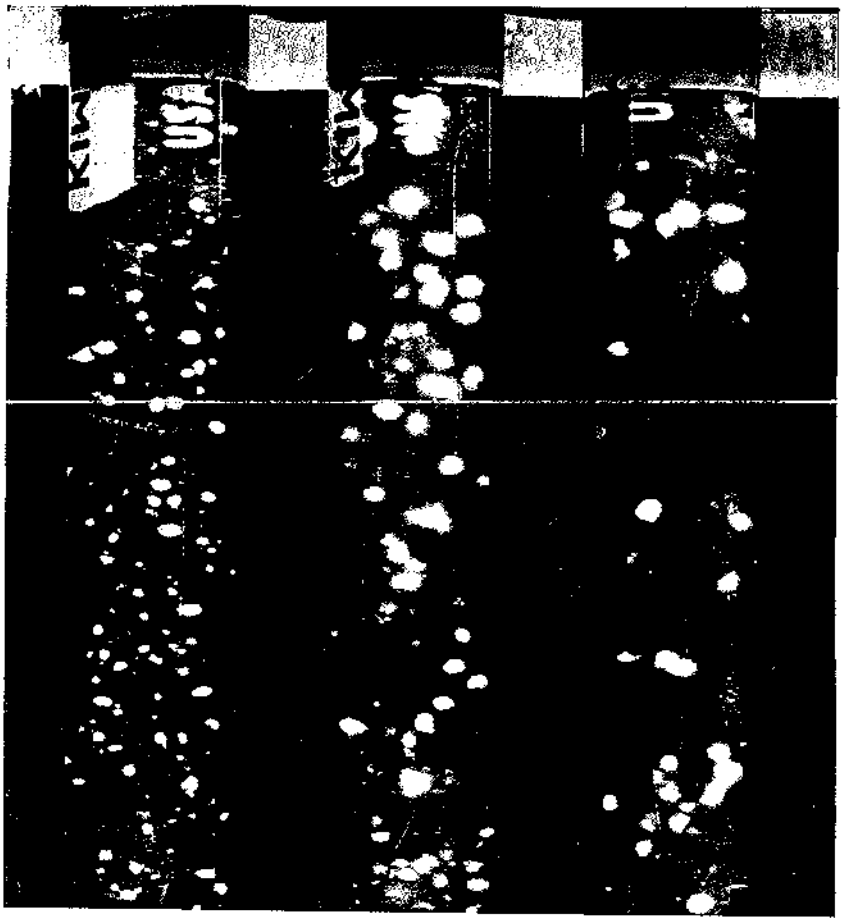


Plate 2

A. 0.18% w/v Agarose
Inoculum = 100 cells/tube
Agarose allowed to gel at room temperature

B. 0.18% w/v Agarose
Inoculum = 600 cells/tube
Agarose allowed to gel at room temperature

C. 0.22% w/v Agarose
Inoculum = 1000 cells/tube
Agarose allowed to gel at room temperature

D. 0.22% w/v Agarose
Inoculum = 600 cells/tube
Agarose cooled rapidly in ice.

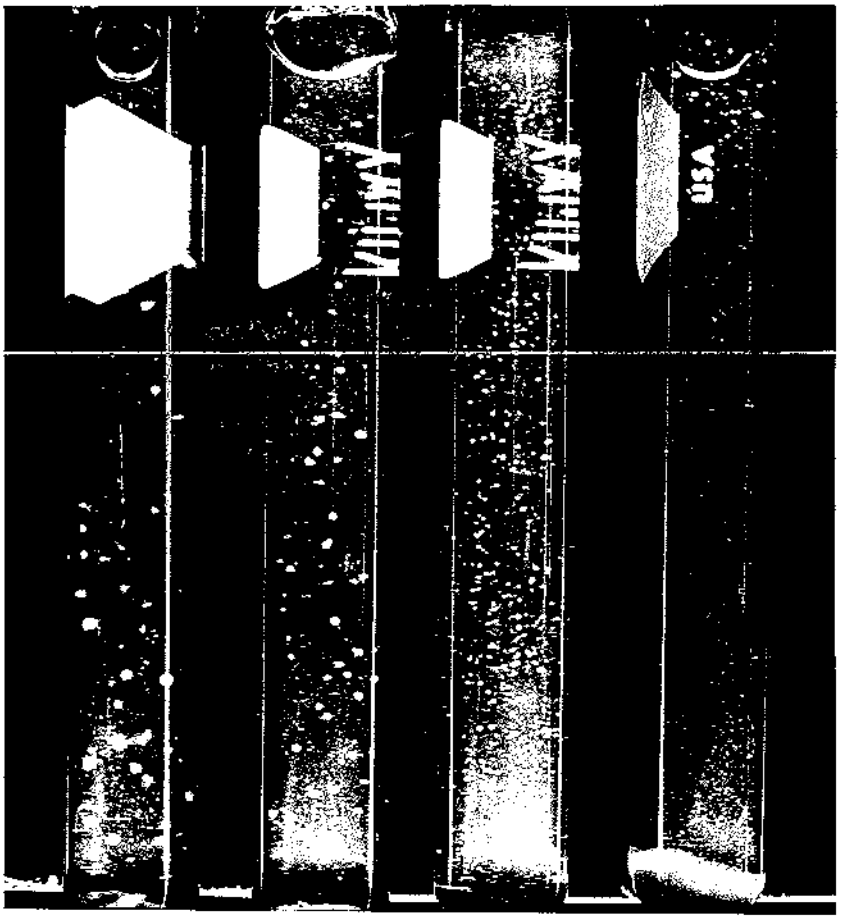

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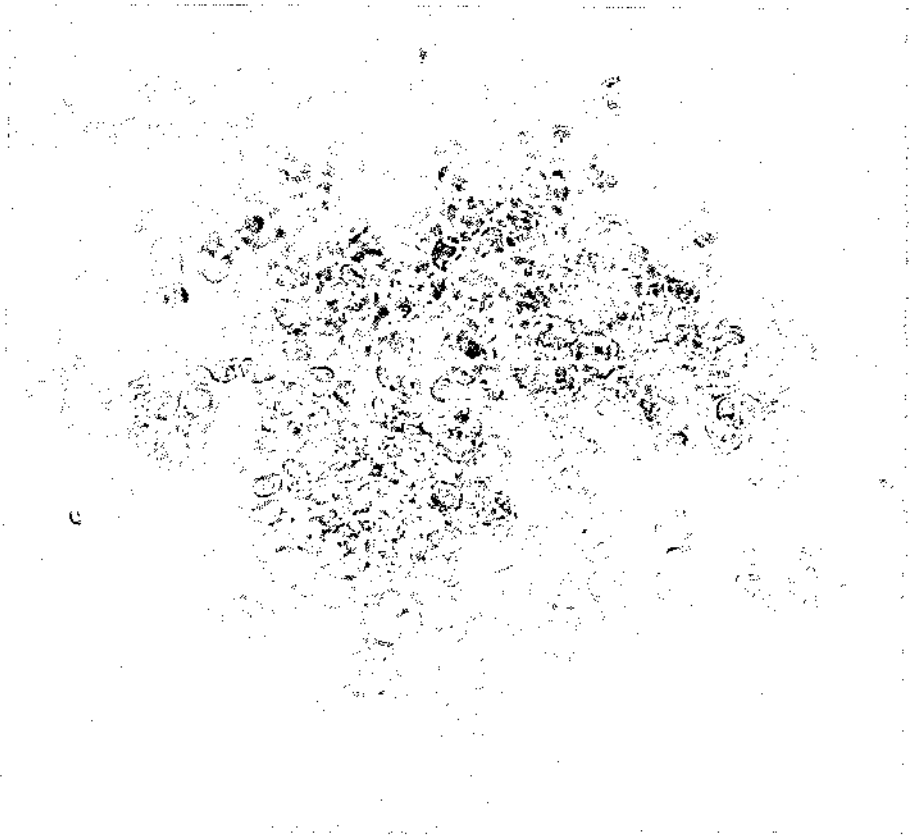
Plate 3 Microscopic Appearance of Hast/87/MUGU/68
Colony suspended in agarose matrix after 8 days incubation at 37°C.



4.3 The Effect of Redox Potential and L-Cysteine Concentration on Culture Growth and Trophozoite Attachment in vitro.

Multiplication of G. intestinalis trophozoites in complex media such as TY1-S-33 is absolutely dependant upon the addition of reducing agents (Gillin and Diamond, 1981). In TY1-S-33, these reducing agents are L-cysteine; (0.15% w/v) and L-ascorbic acid; (0.02% w/v).

During culture growth in vitro, trophozoites demonstrate a tendency to attach to the walls of plastic and glass culture vessels. The L-cysteine requirement for growth in TY1-S-33 is quite specific while a number of thiol reducing agents have been shown to support trophozoite attachment. (Gillin and Diamond, 1981; Gillin and Reiner, 1982).

To investigate in more detail the importance of L-cysteine as a component in complex media, the following experiments were carried out.

By incubating experimental cultures in TY1-S-33, where the concentration of L-cysteine had been varied to produce a range of redox potentials (see 3.4.1), the influence of these factors on cell growth and attachment in vitro was determined.

Figs 8 and 9 demonstrate the correlation between reducing conditions, and growth of experimental cultures in TY1-S-33 growth medium.

Both strains examined, (Bris/83/HEPU/106 and Hast/87/MUGU/68) failed to grow in the absence of L-cysteine. At L-cysteine concentrations below that of complete or 'non-experimental' growth medium (0.15% w/v L-cysteine); culture growth was supported, but at a reduced rate in both strains. As the concentration of L-cysteine was elevated and the redox potential decreased; the growth rates of experimental culture increased to reach maximal levels at 0.15-0.25% w/v L-cysteine.

Figure 8:The Effect of Reducing Conditions on Growth of Strain Hast/87/MUGU/68.

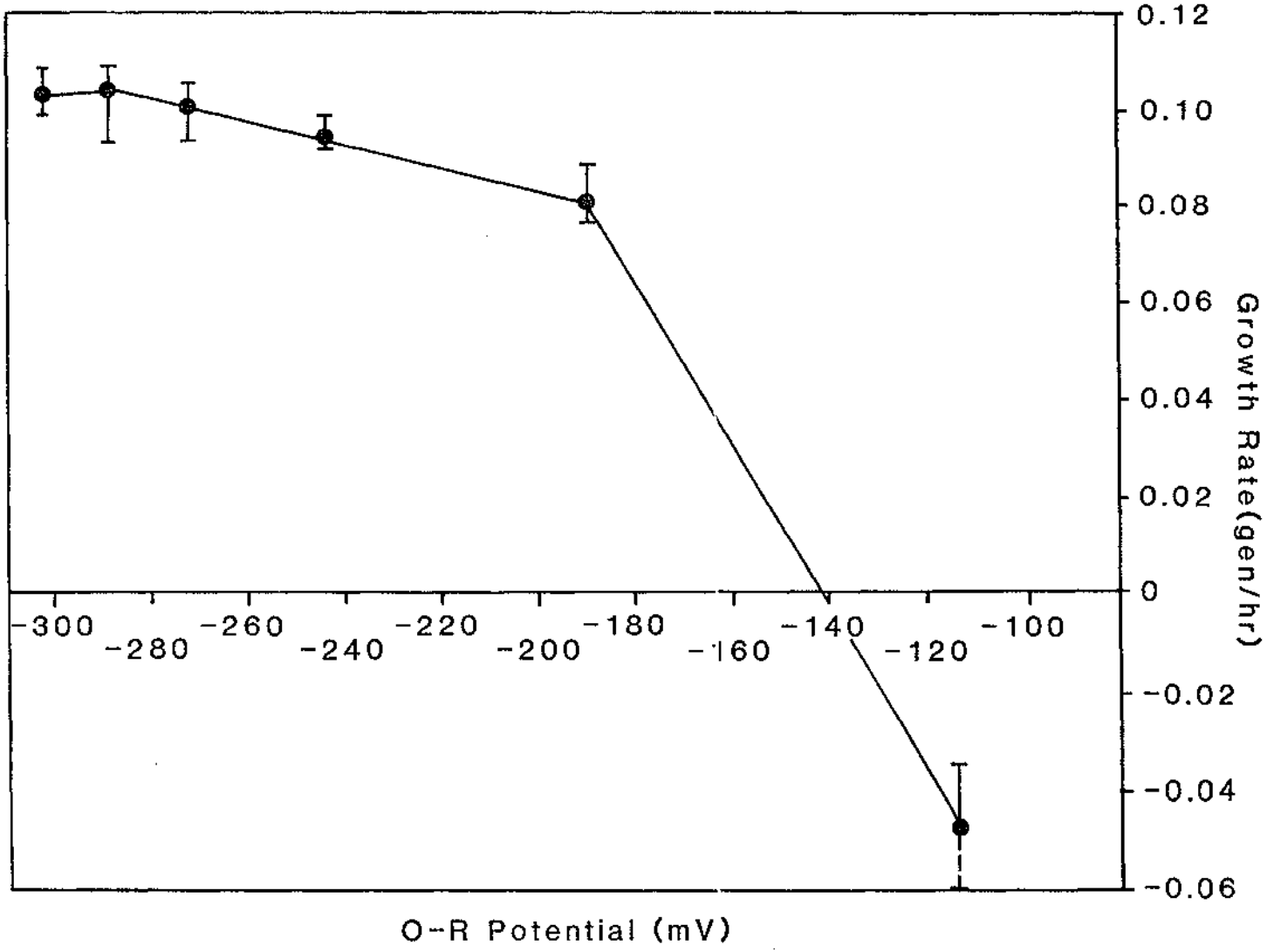
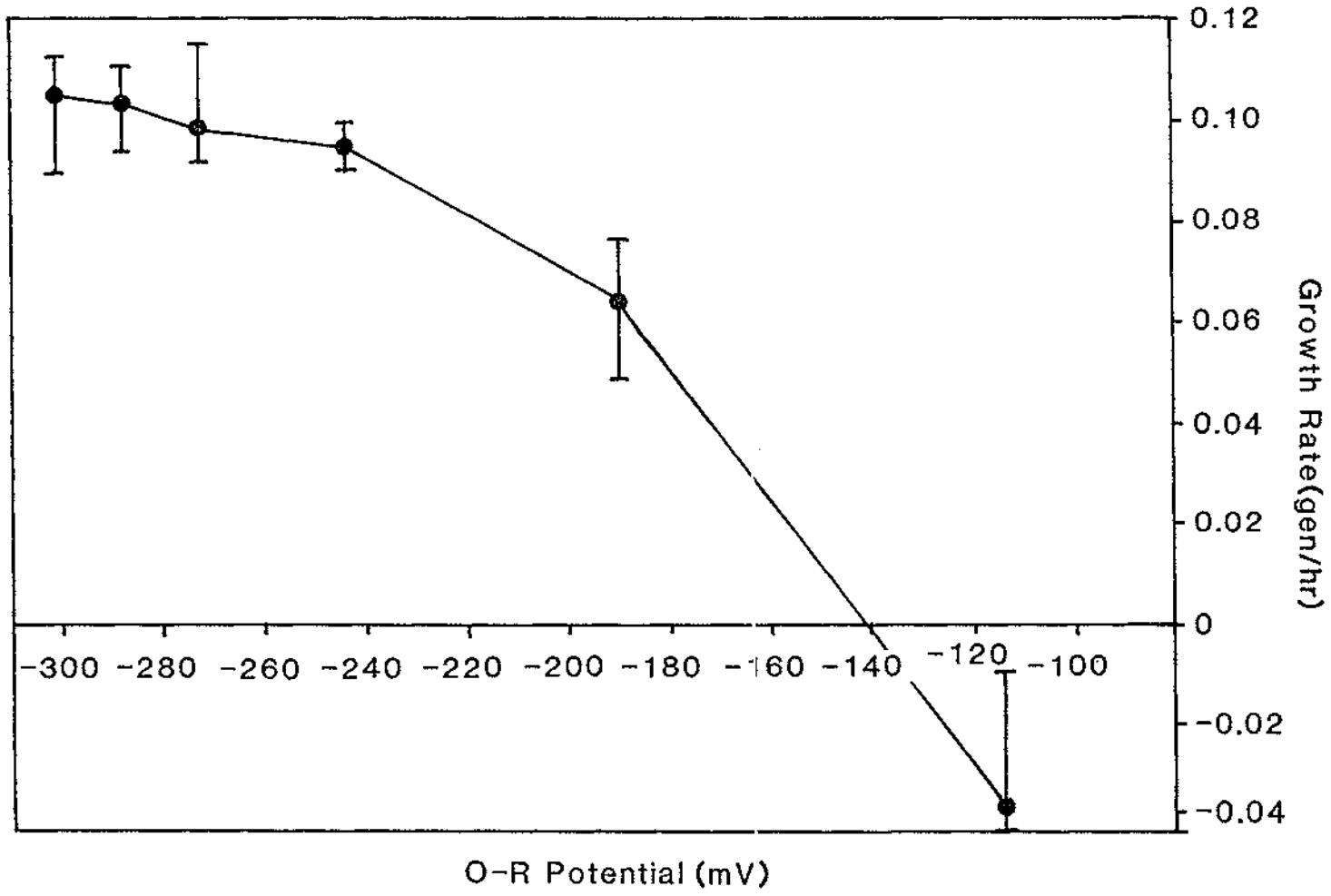


Figure 9:The Effect of Reducing Conditions on Growth of Hast/87/MUGU/68.



The pattern of trophozoite attachment in complete TY1-S-33 is demonstrated in Figs 10 and 11. (See Appendix).

In both Bris/83/HEPU/106 and Hast/87/MUGU/68 cultures, a large proportion of the total trophozoite population attached to the walls of culture tubes very rapidly. This attachment was most rapid during the 30-90 minutes following culture establishment. Under optimal culture conditions (ie complete TY1-S-33 growth medium at 37^{o}C), a high level of trophozoite attachment was achieved and maintained. (see Figs 2 and 3).

Figs 12 and 13 and Tables IV - V demonstrate the strong dependance of this attachment process on reducing conditions.

In the absence of L-cysteine, more than 50% of Bris/83/HEPU/106 and Hast/87/MUGU/68 trophozoites remained unattached or 'free' with increasing L-cysteine concentrations and accompanying reduced redox potentials, the proportion of trophozoites attached increased to the maximum levels of 85-95% of that obtained in complete TY1-S-33. Even as L-cysteine levels were elevated above 0.15% w/v, the 'trophozoite attachment percentage' did not exceed this maximum.

Figure 10: Attachment of Bris/83/HEPU/106 in vitro.

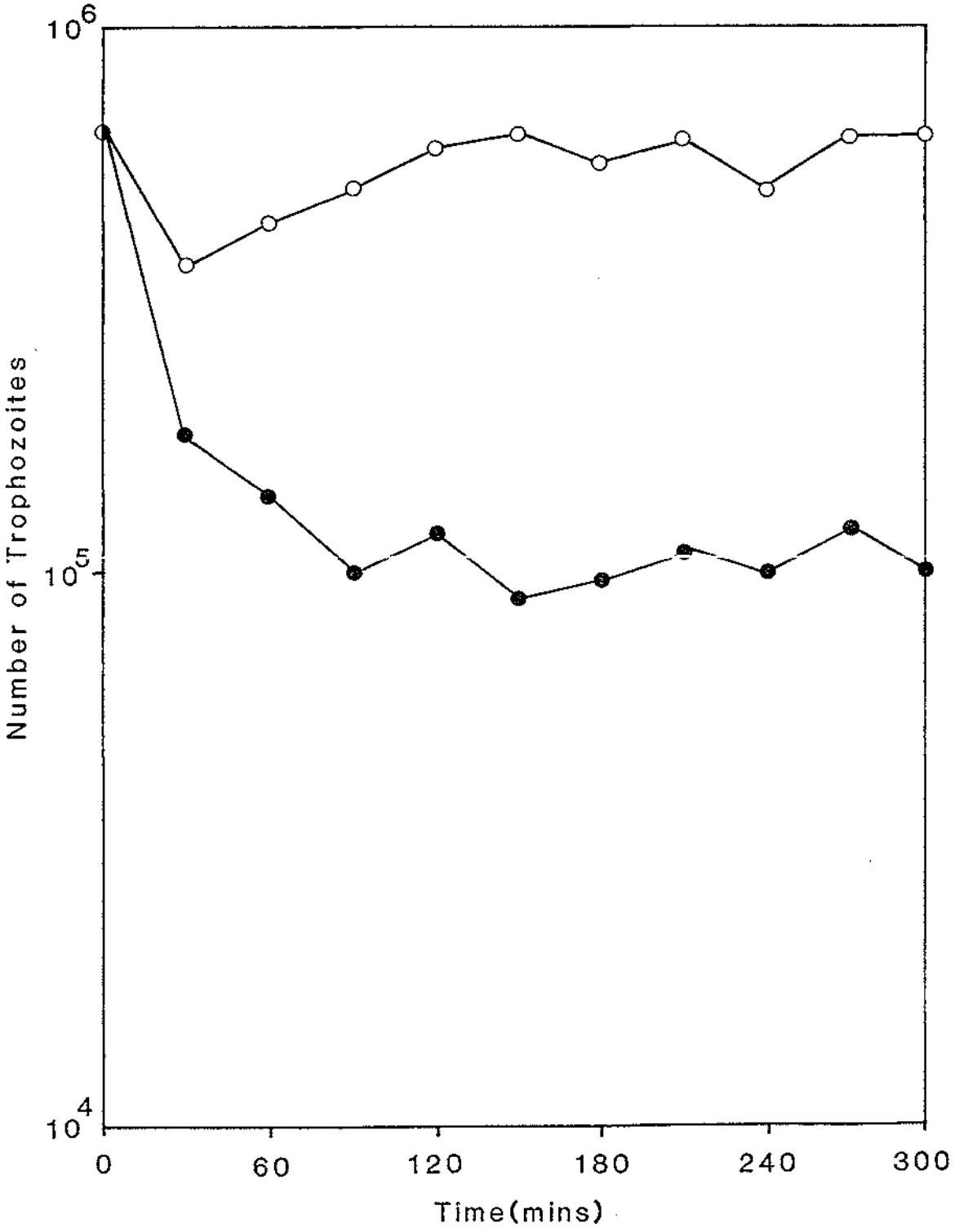
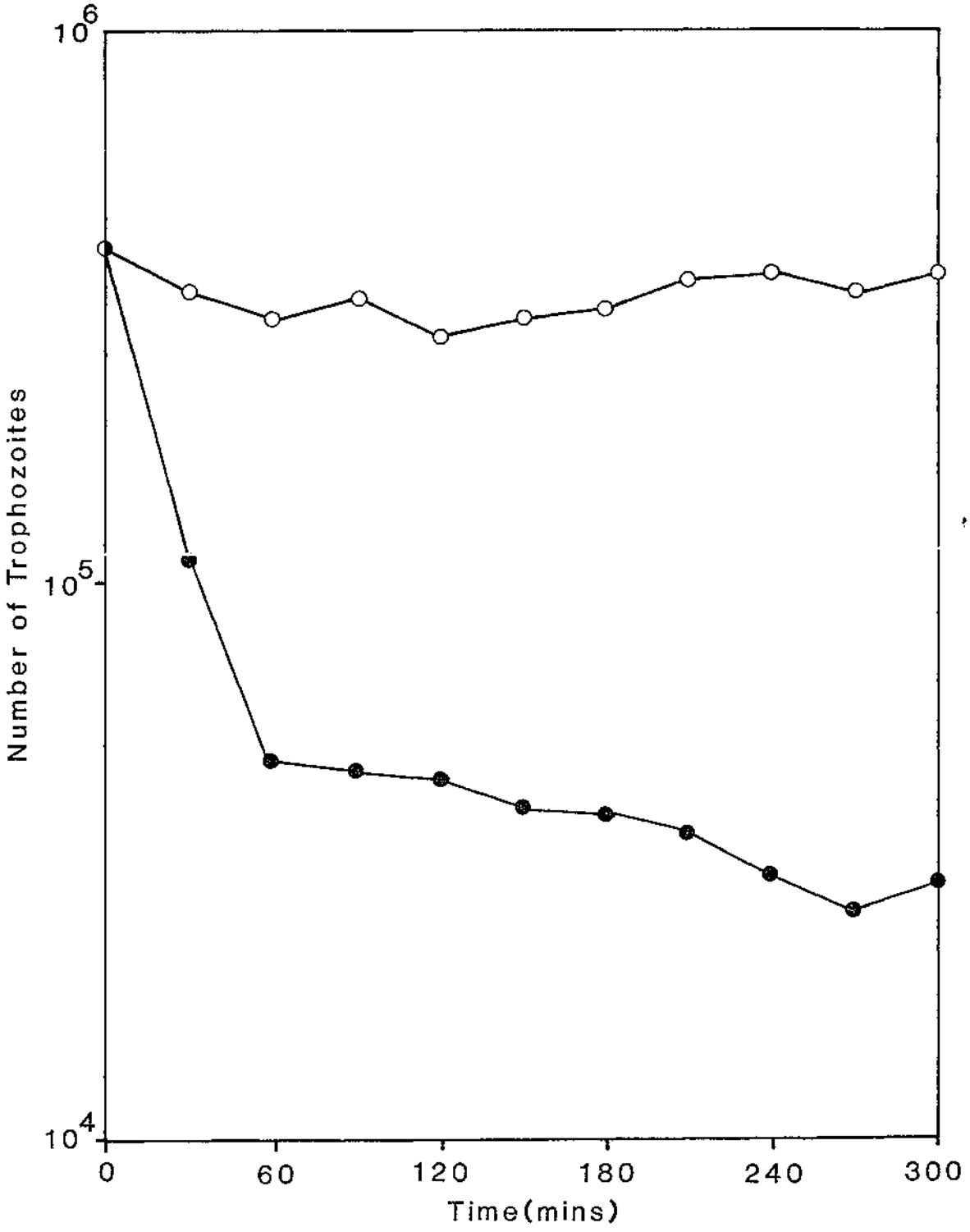


Figure 11: Attachment of Hast/87/MUGU/68 *in vitro*.



In Figs 12 and 13 the following legend applies:

■ 'Free' or non-adherent trophozoites at 0.00% w/v L-cys; -114.7 mV

□ 'Attached' or adherent trophozoites at 0.00% w/v L-cys; -114.7 mV

● 'Free' or non-adherent trophozoites at 0.05% w/v L-cys; -191.5 mV

○ 'Attached' or adherent trophozoites at 0.05% w/v L-cys; -191.5 mV

▲ 'Free' or non-adherent trophozoites at 0.10% w/v L-cys; -243.6 mV

△ 'Attached' or adherent trophozoites at 0.10% w/v L-cys; -243.6 mV

◆ 'Free' or non adherent trophozoites at 0.15% w/v L-cys; -272.4 mV

◇ 'Attached' or adherent trophozoites at 0.15% w/v L-cys; -272.4 mV

▼ 'Free' or non-adherent trophozoites at 0.20% w/v L-cys; -289.8 mV

▽ 'Attached' or adherent trophozoites at 0.20% w/v L-cys; -289.8 mV

★ 'Free' or non-adherent trophozoites at 0.25% w/v L-cys; -302.1 mV

☆ 'Attached' or adherent trophozoites at 0.25% w/v L-cys; -302.1 mV

Figure 12: The Effect of Reducing Conditions on Bris/83/HEPU/106 Attachment.

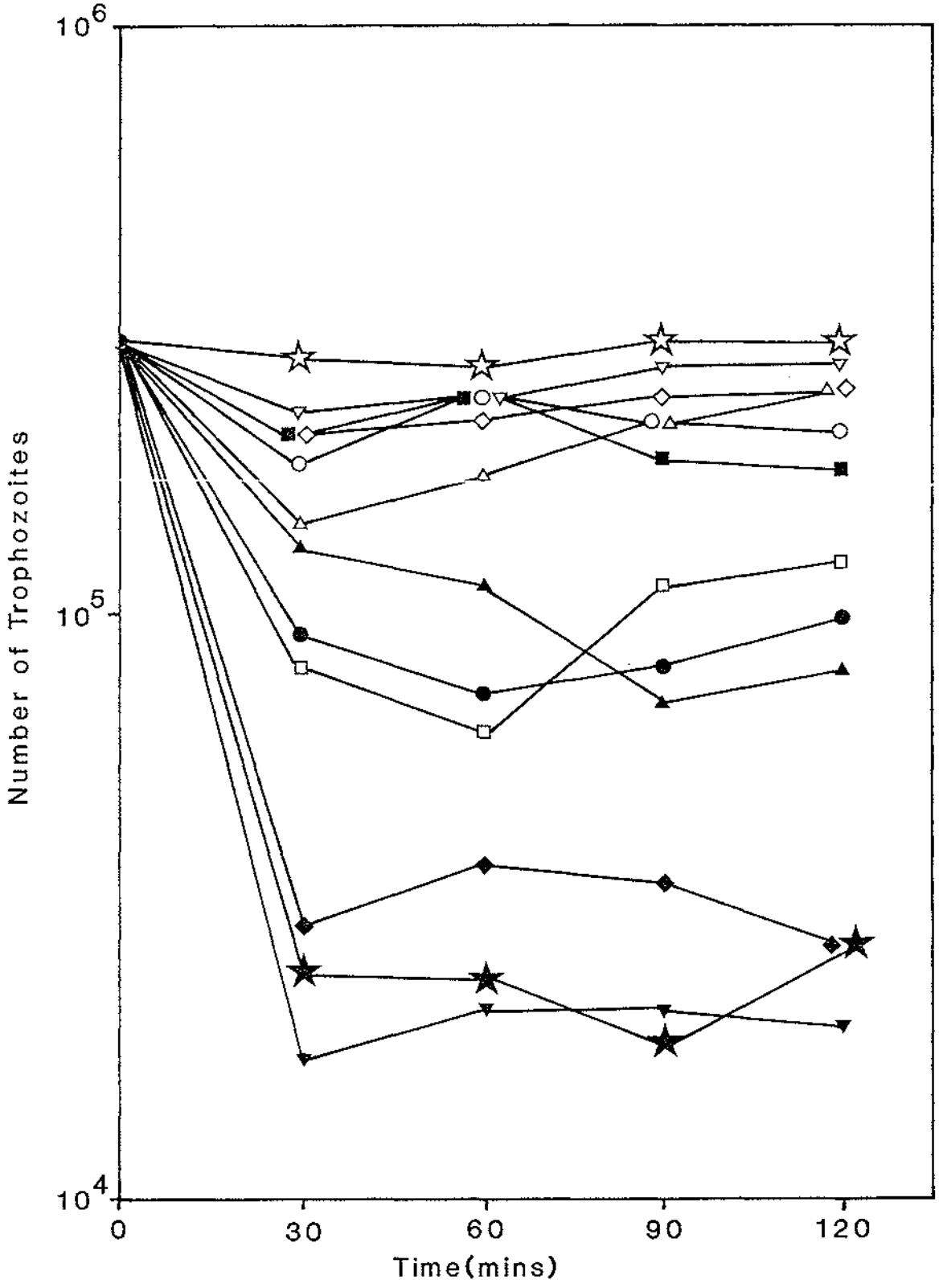


Table IV: The Effect of Reducing Conditions on Bris/83/HEPU/106 Attachment

L-cysteine conc (% w/v)	O-R Potential (mV)	Trophozoite Attachment (%)[a] at T 120 mins
0.00	-114.7	41.4
0.05	-191.5	67.1
0.10	-243.6	74.7
0.15	-272.4	89.6
0.20	-289.8	90.6
0.25	-302.1	92.9

a % Attachment = $\frac{\text{Attached Trophozoites}}{\text{Free + Attached Trophozoites}} \times 100$

Trophozoite Attachment percentages expressed as the mean of 2-3 determinations.

Figure 13: The Effect of Reducing Conditions on Hast/87/MUGU/68 Attachment.

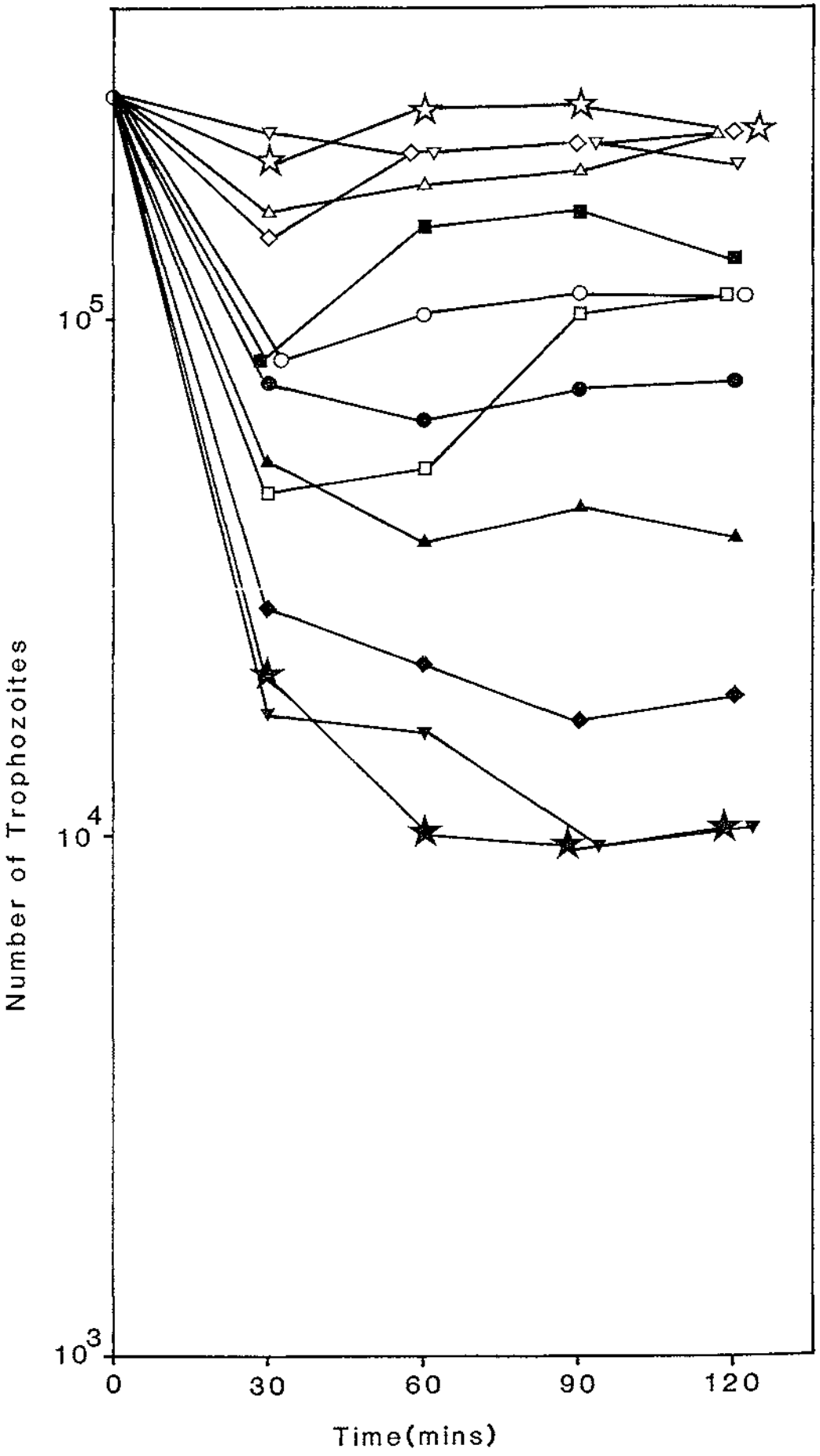


Table V: The Effect of Reducing Conditions on Hast/87/MUGU/68 Attachment.

L-cysteine conc (% w/v)	O-R Potential (mV)	Trophozoite Attachment (%)[a] at T 120 mins
0.00	-114.7	45.8
0.05	-191.5	59.1
0.10	-243.6	86.5
0.15	-272.4	92.4
0.20	-289.8	96.0
0.25	-302.1	95.5

4.4 Sensitivity of G. intestinalis Trophozoites to Elevated Dissolved Oxygen Levels in TY1-S-33 Growth Medium.

G. intestinalis trophozoites are oxygen-sensitive (Gillin and Diamond, 1981). For this reason vessels used for trophozoite culture are filled completely with liquid growth medium rich in reducing agents, minimising exposure of trophozoites to oxygen.

The following experiments were designed to investigate the extent of this oxygen sensitivity by exposing trophozoites to a range of Dissolved Oxygen (D.O.) levels in TY1-S-33 growth medium at 37°C. (The D.O. level of freshly prepared TY1-S-33 = 0.4-0.8 ppm at 37°C).

The responses of G. intestinalis trophozoites to increased D.O. levels are plotted in Figs 14-21 and Tables VI - VII.

Bris/83/HEPU/106 and Hast/87/MUGU/68 demonstrated similar sensitivities to elevated oxygen tensions.

At 4.0 ppm D.O.; a slow decline in the numbers of viable trophozoites was observed in both strains. This loss of viability was more rapid under 6.0 ppm D.O. conditions. As D.O. levels were increased to 8.0 ppm, a lag phase in which culture viability was maintained, and a subsequent phase of experimental killing were recorded. The duration of this 'lag phase' was 3-4 hours, followed by a rapid loss of culture viability over 2-3 hours. This was the case for both Bris/83/HEPU/106 and Hast/87/MUGU/68.

Trophozoites of both strains were markedly sensitive to exposure to 12.0 ppm D.O. Exponential killing of trophozoites commenced almost immediately after inoculation of experimental media. The lag phase, when present, was only of up to 30 minutes duration

Figure 14: Sensitivity of Bris/83/HEPU/106 to 4.0 ppm dissolved Oxygen in TY1-S-33 Growth Medium.

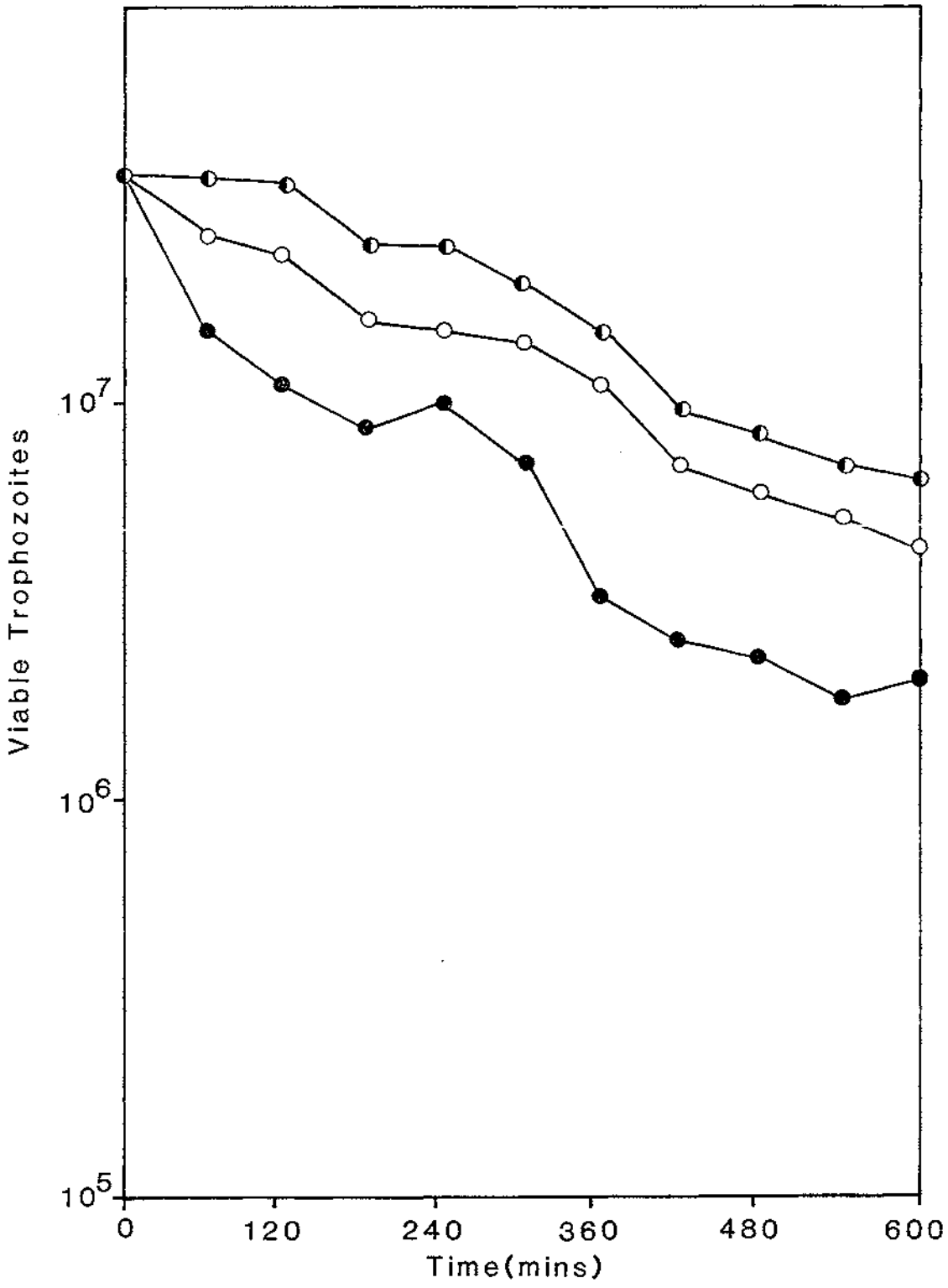


Figure 15: Sensitivity of Bris/83/HEPU/106 to 6.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium.

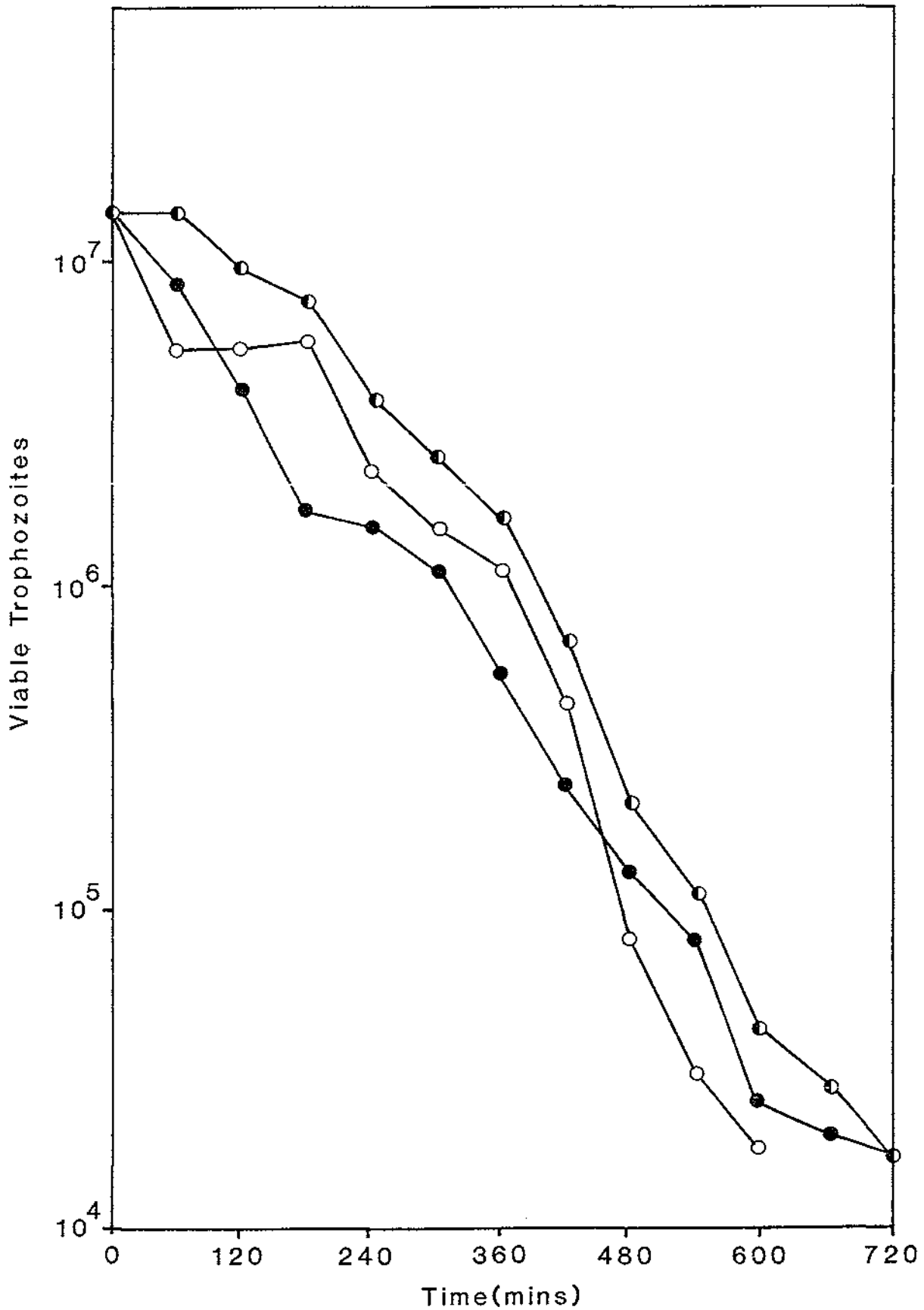


Figure 16: Sensitivity of Bris/83/HEPU/106 to 8.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium.

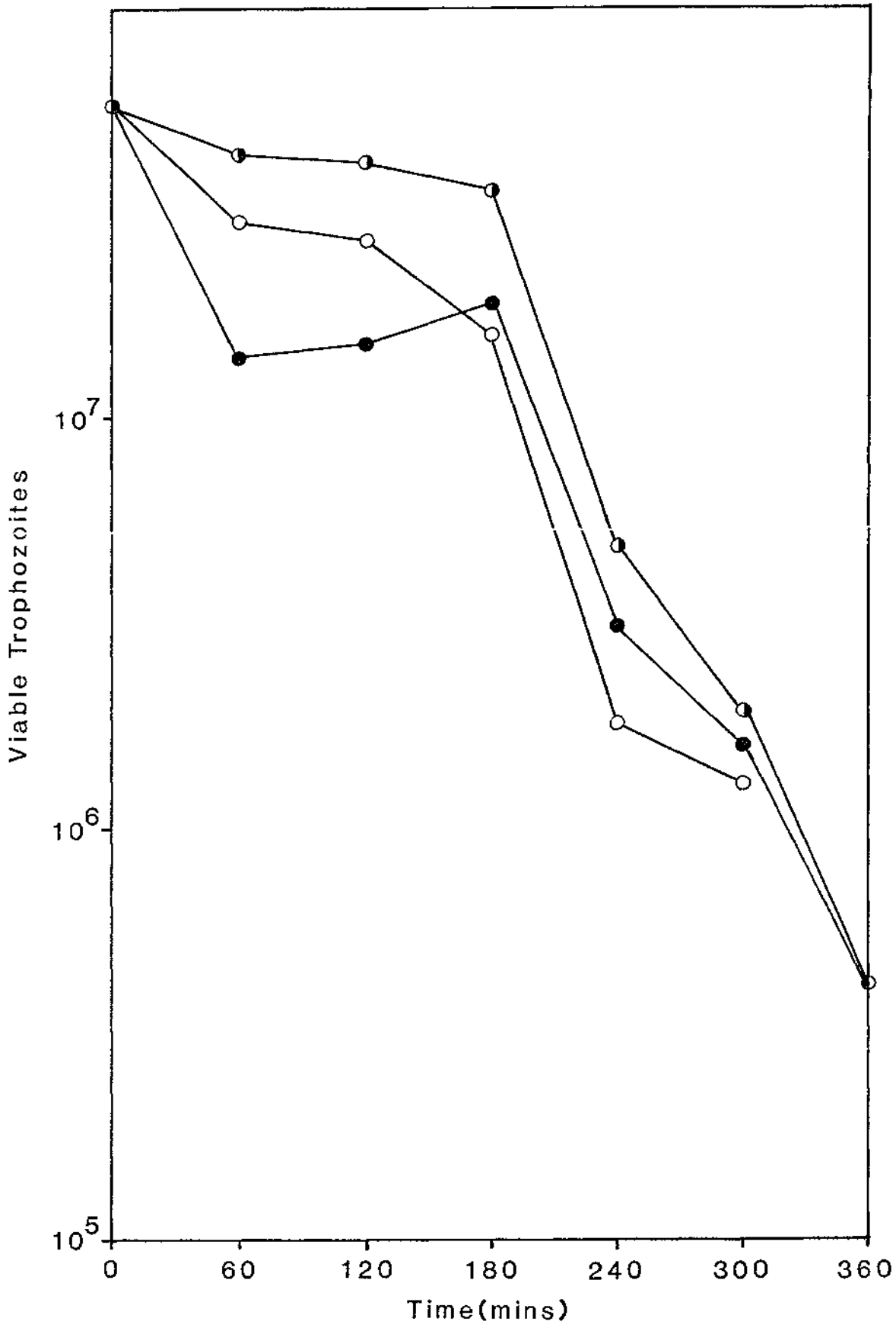


Figure 17: Sensitivity of Bris/83/HEPU/106 to 12.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium.

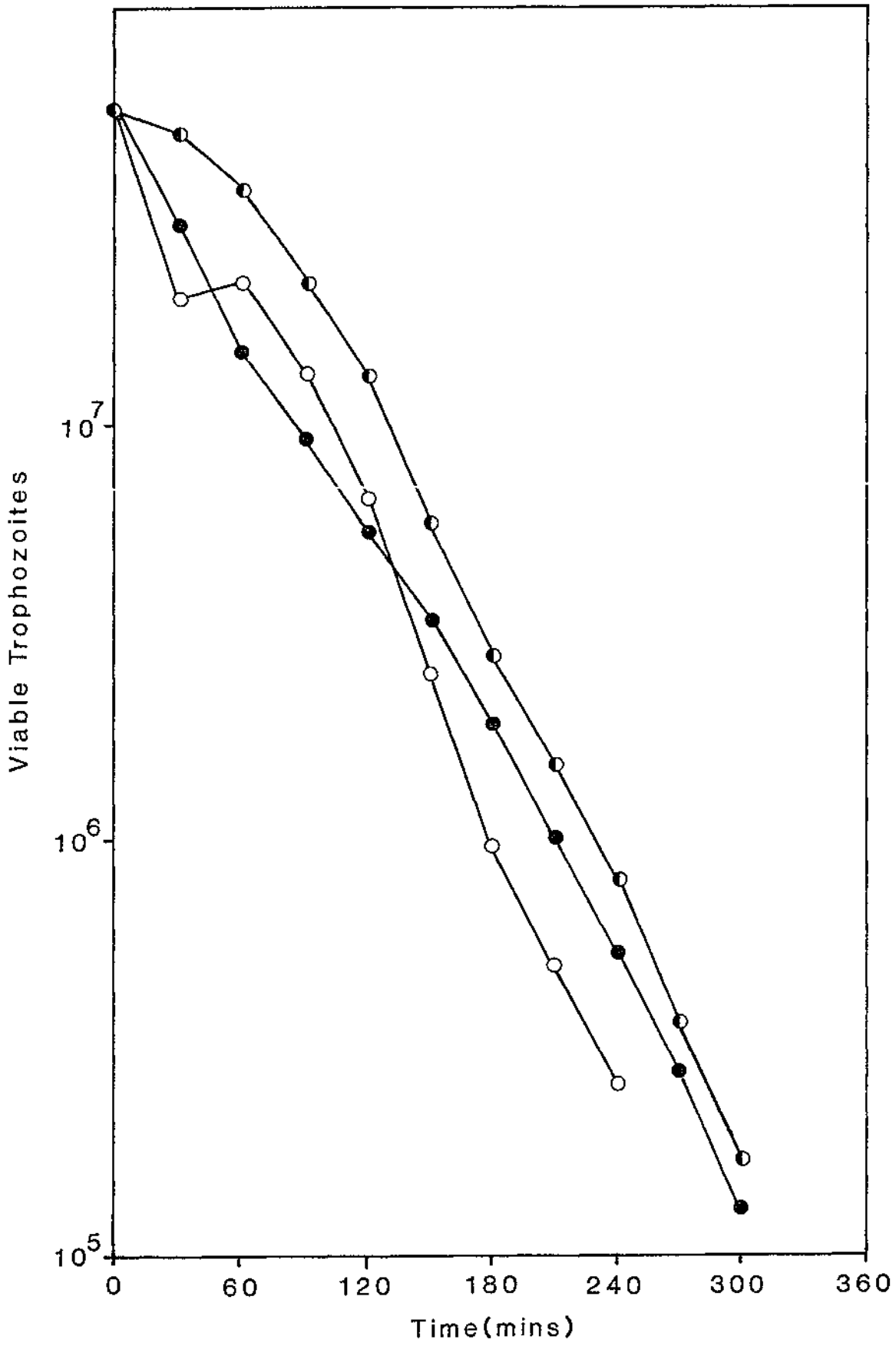


Figure 18: Sensitivity of Hast/87/MUGU/68 to 4.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium.

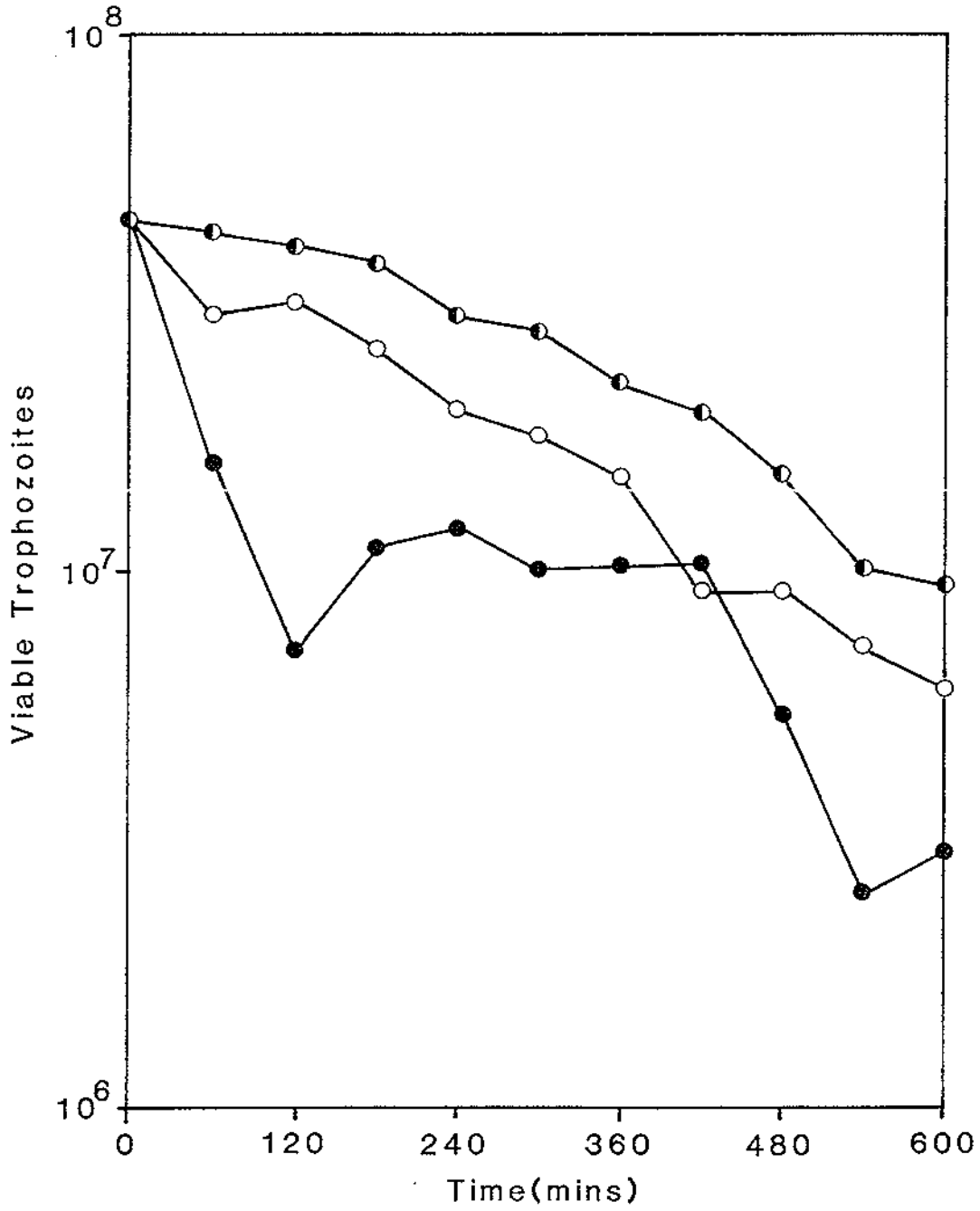


Figure 19: Sensitivity of Hast/87/MUGU/68 to 6.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium.

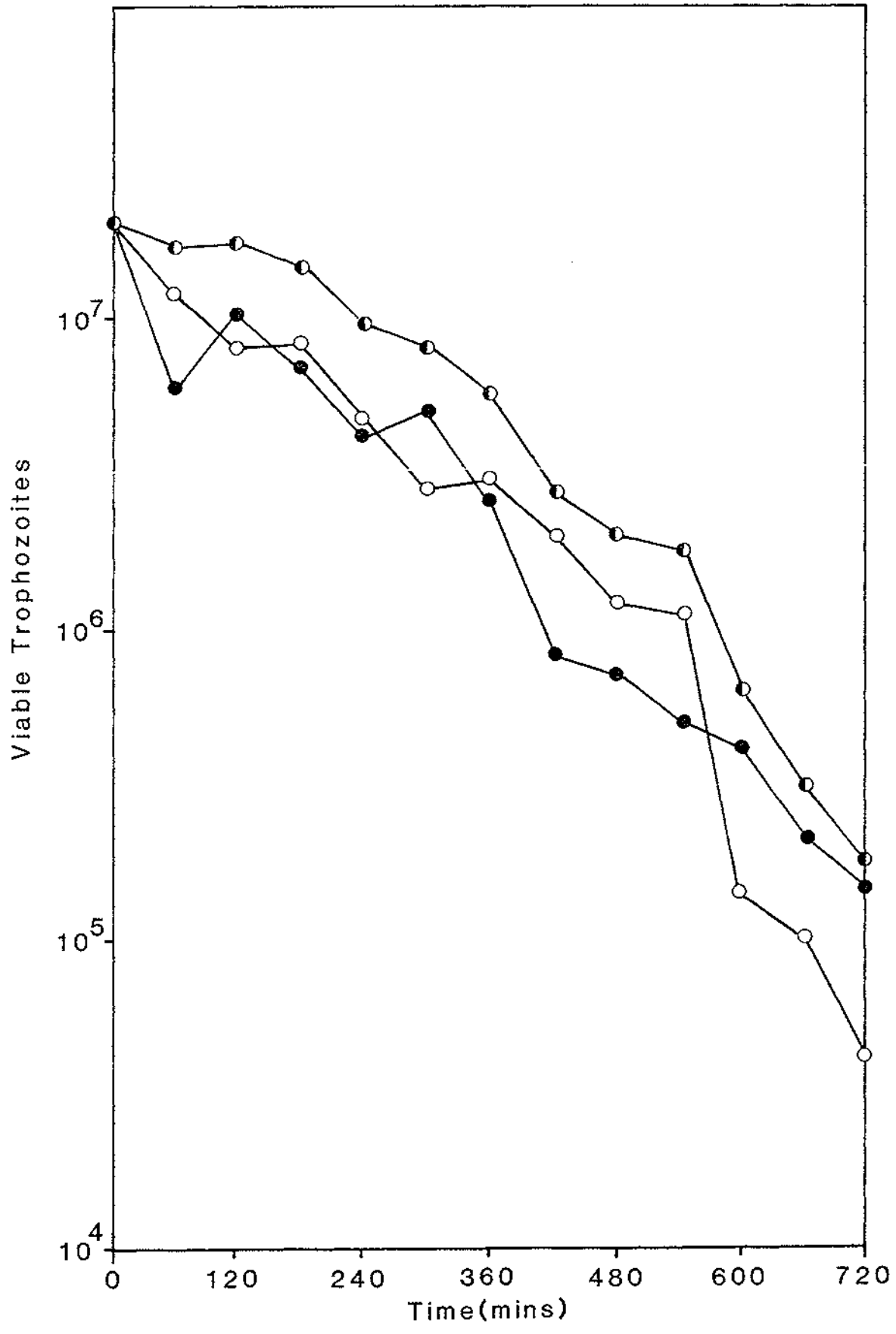


Figure 20: Sensitivity of Hast/87/MUGU/68 to 8.0 ppm dissolved Oxygen in TY1-S-33 Growth Medium.

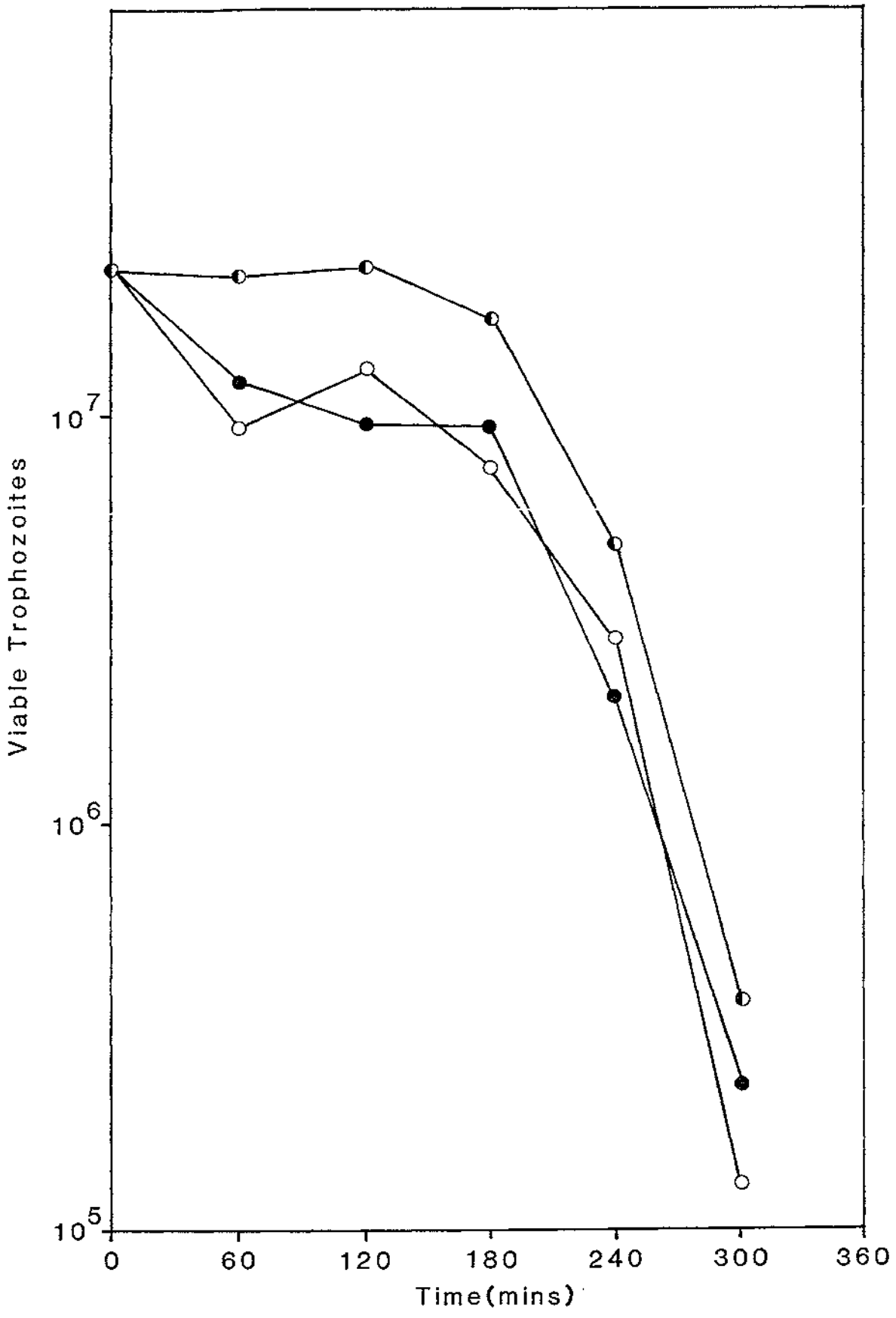
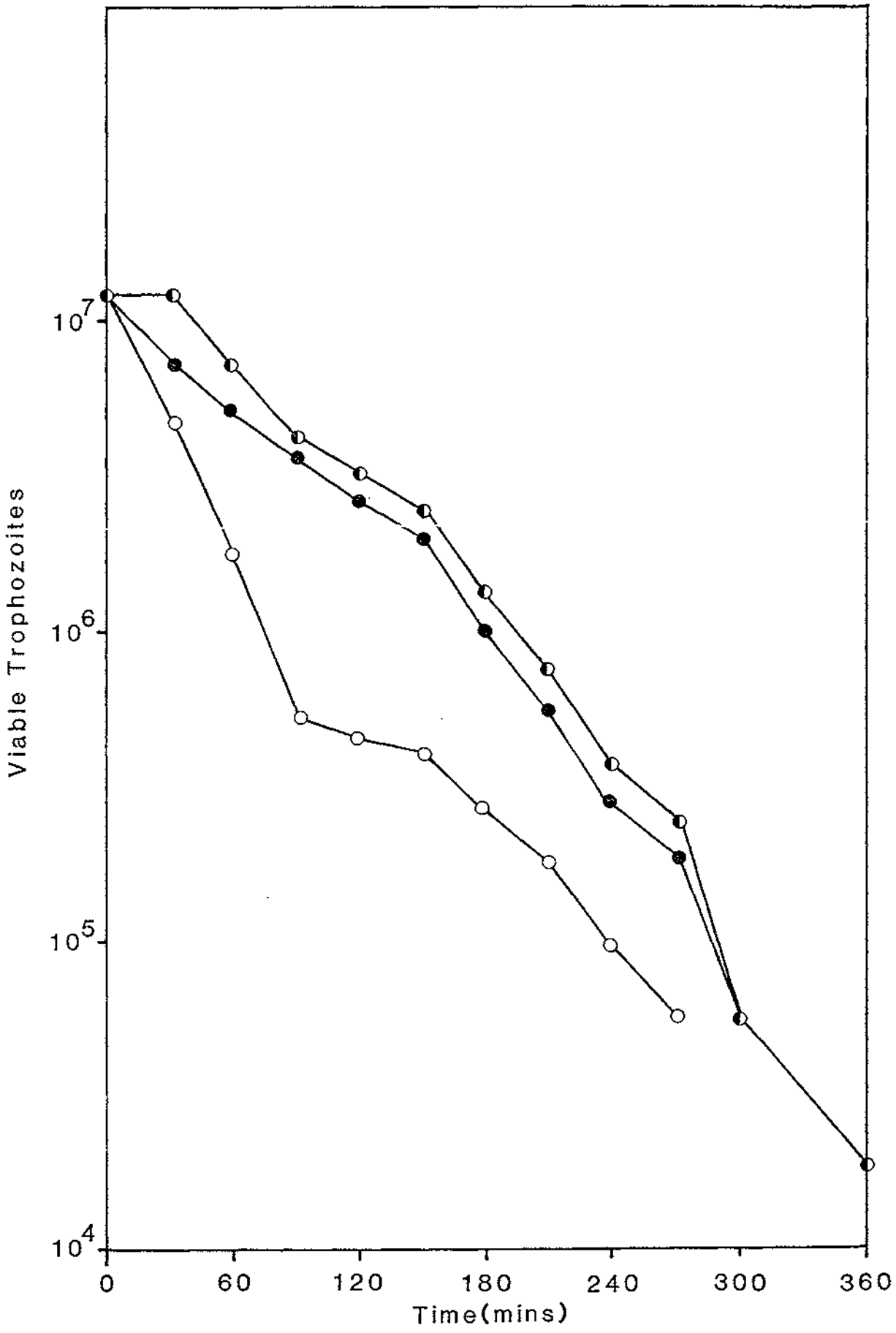


Figure 21: Sensitivity of Hast/87/MUGU/68 to 12.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium.



The Effect of Temperature on the Oxygen Sensitivity of Trophozoites.

To further define the oxygen-sensitivity of trophozoites; Bris/83/HEPU/106 was exposed repeatedly to elevated D.O. levels (8.0-12.0 ppm), at temperatures below 37°C. In this way the effects of increased D.O. on trophozoite survival could be determined with respect to temperature.

Figs 22-25 and Table VIII show the reduction in oxygen-sensitivity of Bris/83/HEPU/106 trophozoites under reduced temperatures.

At 8.0 ppm D.O. and 30°C; the lag phase which preceded exponential killing of trophozoites was 6-8 hours long as opposed to the lag phase of 3-4 hours observed with the same D.O. levels at 37°C. The sensitivity of Bris/83/HEPU/106 trophozoites to 12.0 ppm D.O. also diminished with reduced temperatures. At 30°C, a 1-2 hour lag phase preceded a loss in culture viability. At 12.0 ppm D.O. and 20°C; over a time-period comparable to that of culture exposure to the same D.O. level at 37°C; no exponential killing was observed.

All results expressed are the mean of 2-3 determinations.

Figure 22: Survival of Bris/83/HEPU/106 at Reduced Temperatures in TY1-S-33 Growth Medium.

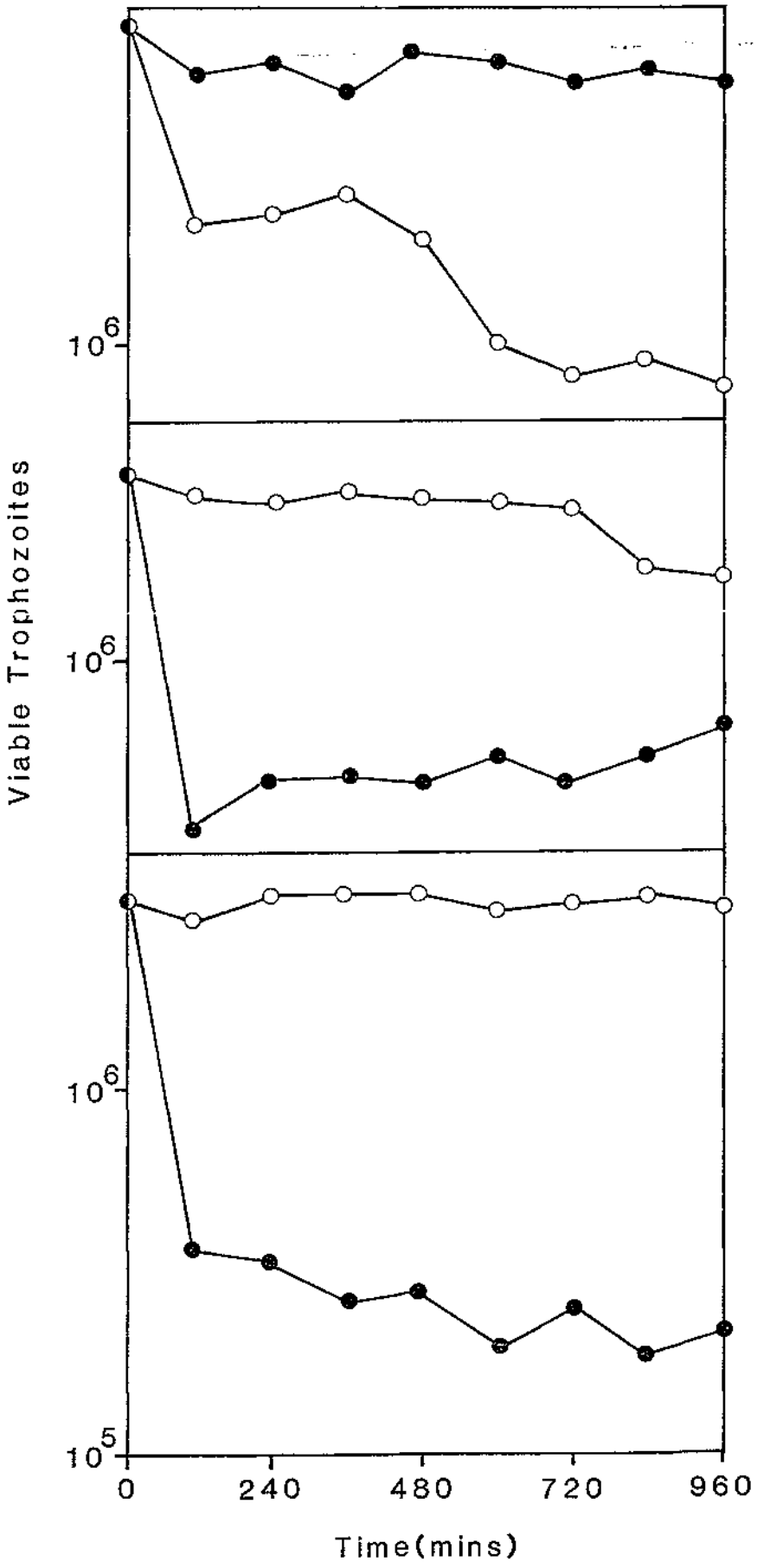


Figure 23: Sensitivity of Bris/83/HEPU/106 to 8.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium at 30°C.

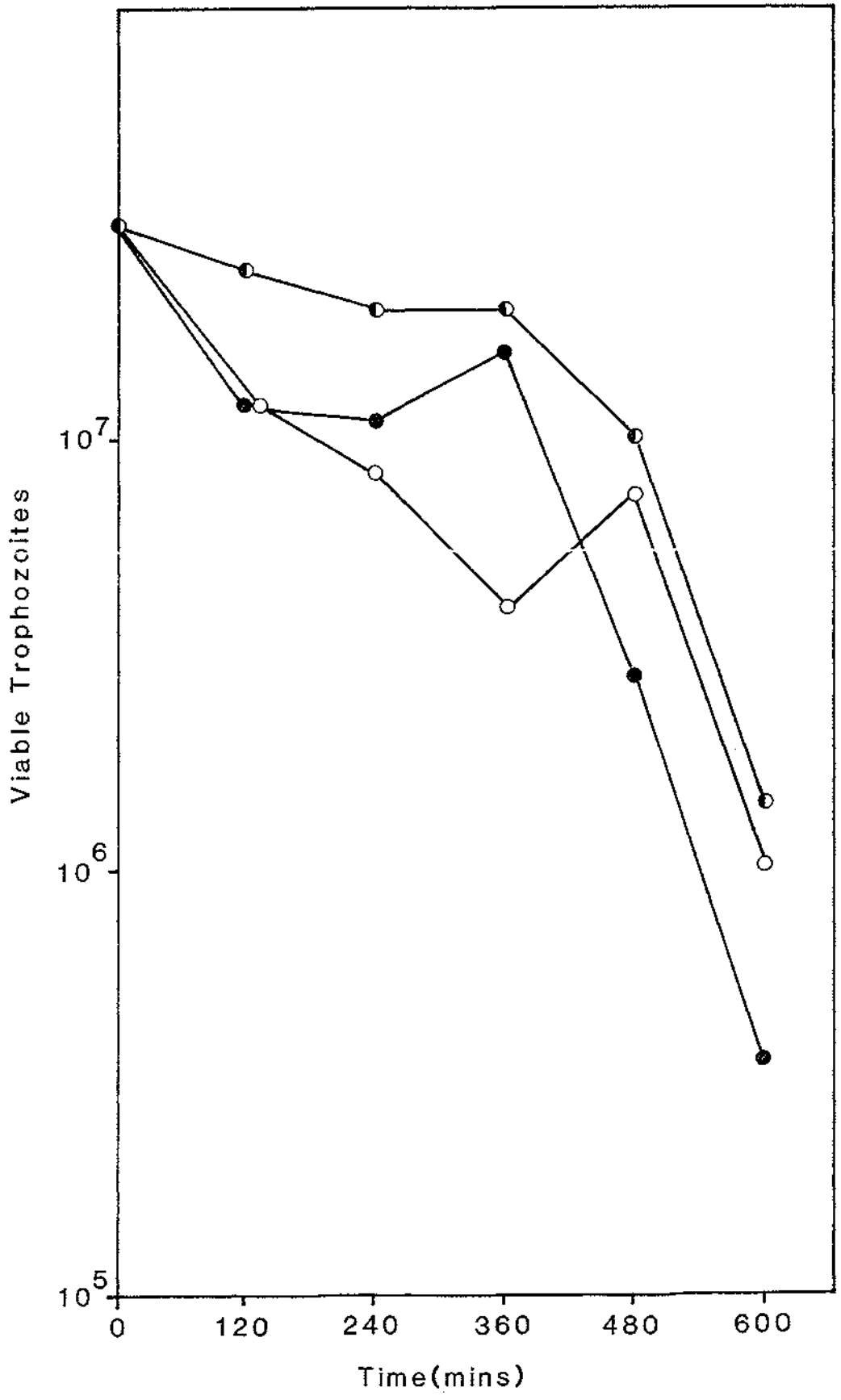


Figure 24: Sensitivity of Bris/83/HEPU/106 to 12.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium at 30°C.

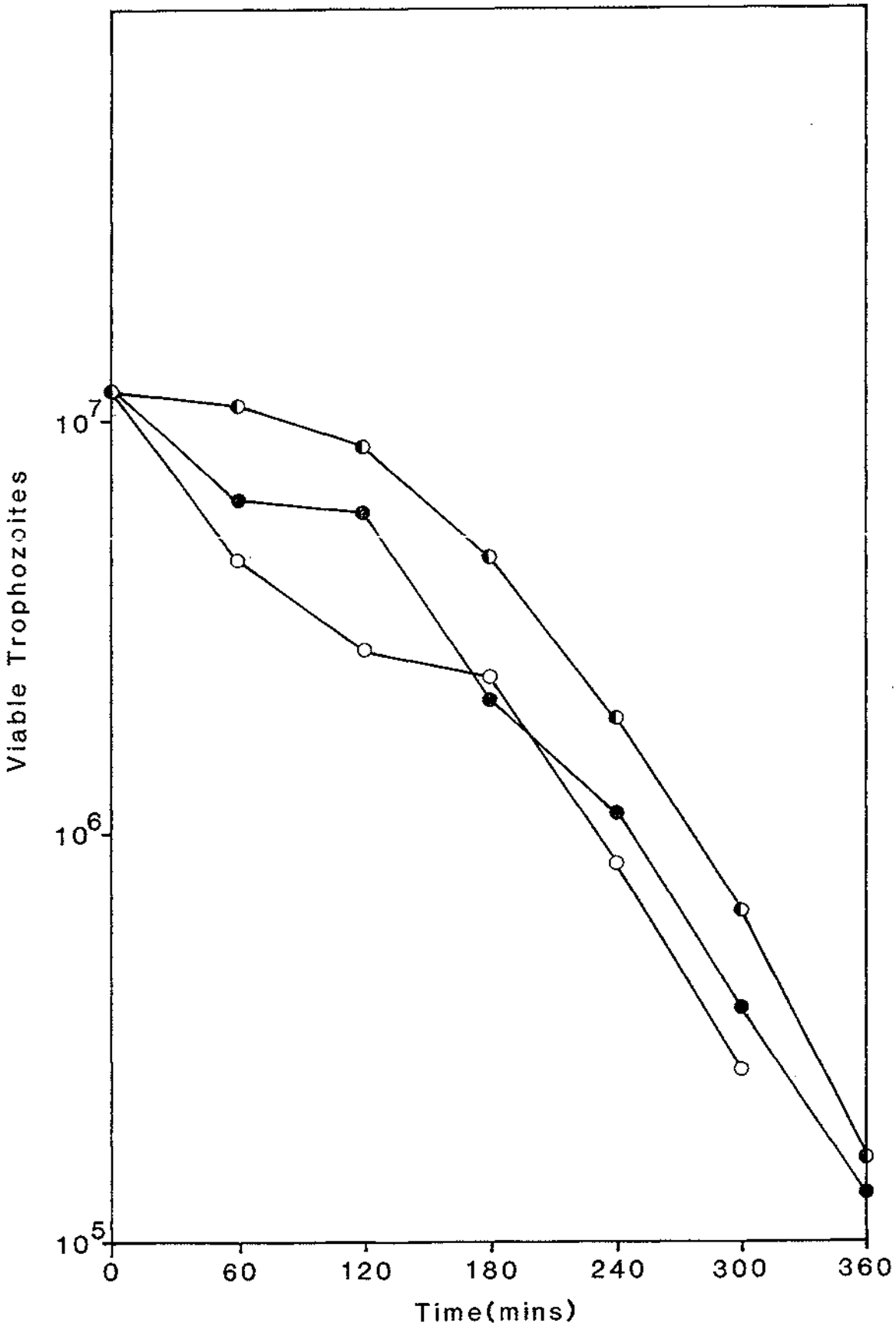


Figure 25: Sensitivity of Bris/83/HEPU/106 to 12.0 ppm Dissolved Oxygen in TY1-S-33 Growth Medium at 30°C.

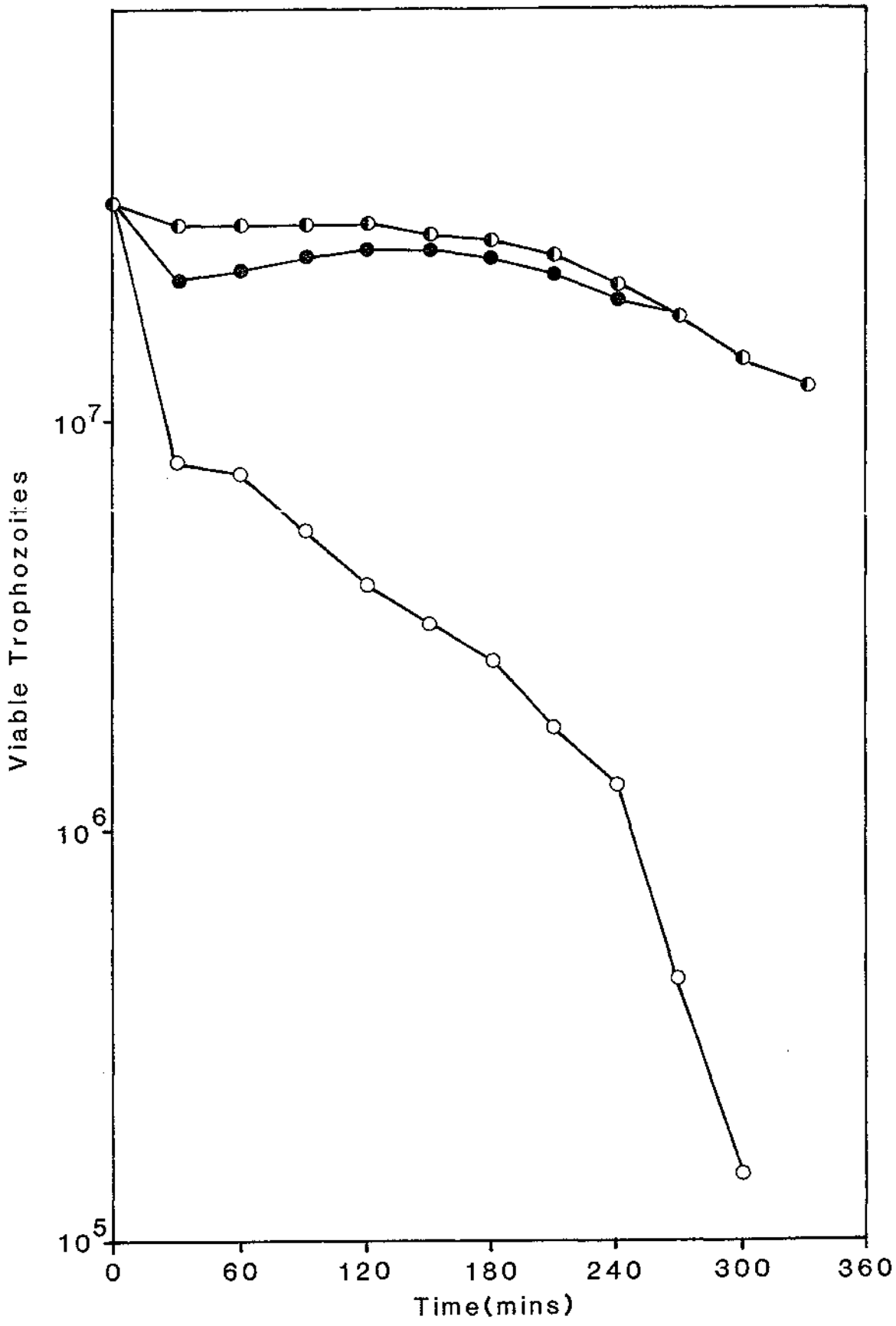
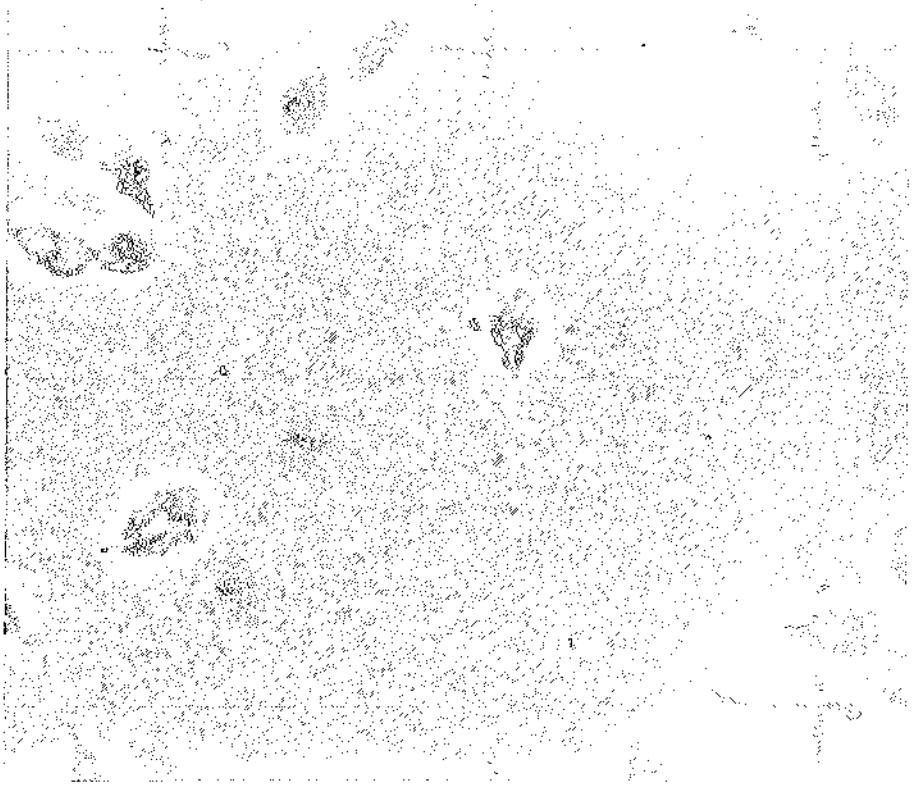


Plate 4 Gross morphology of healthy Bris/83/HEPU/106 trophozoites after cultivation in TY1-S-33.

Plate 5 Morphological distortion of trophozoites after exposure to 8.0 ppm D.O. at 37°C. Note incomplete division (top left) and clumping (bottom right) of trophozoites.



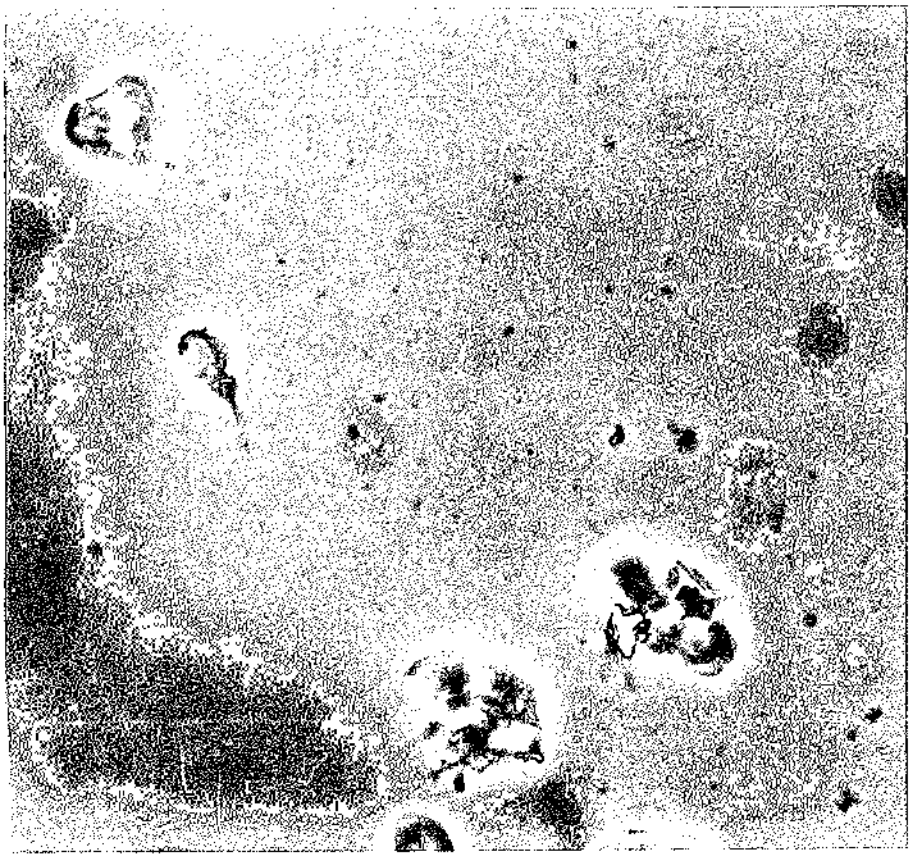


Table VI: Killing of Bris/83/HEPU/106 Trophozoites by Exposure to Elevated D.O. Levels in TY1-S-33 Growth Medium at 37°C.

D.O. Level ppm	T½ Killing (mins)
4.0	324
6.0	192
8.0	186
12.0	72

Table VII: Killing of Hast/87/MUGU/68 Trophozoites by Exposure to Elevated D.O. Levels in TY1-S-33 Growth Medium at 37°C.

D.O. Level ppm	T½ Killing (mins)
4.0	360
6.0	236
8.0	202
12.0	74

T½ Killing = The time required for a 50% decrease in culture viability

Table VIII: The Effect of Incubation Temperature on the Killing of Bris/83/HEPU/106 Trophozoites by Exposure to Elevated D.O. Levels in TY1-S-33 Growth Medium.

D.O. Level (ppm)	Incubation Temperature (°C)	T½ Killing (mins)
8.0	30	380
12.0	30	158
12.0	20	278

4.5 Oxygen Consumption by G. intestinalis Trophozoites.

G. intestinalis is an aerotolerant anaerobe which respires in the presence of oxygen (Jarrol et al; 1981; Lindmark, 1980). Trophozoite suspensions avidly consume oxygen, demonstrating the high affinity of respiratory enzymes for this gas (Weinbach et al; 1980).

The aim of this study was to examine the extent of this oxygen consumption and express it in terms of changes in the Dissolved Oxygen (D.O.) level of trophozoite suspensions.

To ensure the D.O. changes observed were, in fact, due to oxygen consumption by trophozoites, changes in the D.O. level of sterile PBS at 37°C were first determined.

The D.O. level of sterile PBS decreased 1.4 ppm in the first 30 minutes of the assay. The total decrease in D.O. over 5 hours was 1.604 ppm. (See Fig 26).

Changes in the D.O. of trophozoite suspensions are shown in Fig 27. The recorded decrease in the D.O. level of trophozoite suspension 'A' was double that observed in sterile PBS. Trophozoite suspension 'B' with a larger inoculum, produced a decrease in D.O. which was 4-5 fold greater than those observed in PBS controls. Oxygen was consumed by trophozoites at a rate of $3.7\text{-}5.3 \times 10^{-9}$ ppm/cell/hour.

Quinacrine dihydrochloride functions as a flavoantagonist (Hellerman, 1946); and as such, has been shown to inhibit oxygen consumption by G. intestinalis trophozoites (Lindmark, 1980; Weinbach et al; 1980).

The following experiments were designed to investigate the inhibitive effect of Quinacrine on oxygen consumption by Hast/87/MUGU/68 trophozoites.

Since Quinacrine is also a drug which is used for treatment of

giardiasis *in vivo* (Craft *et al*; 1981); and is effective in killing *G. intestinalis in vitro* (Jokipii and Jokipii 1980; Gillin and Diamond, 1981; Smith *et al*; 1982; Boreham *et al*; 1984); it was felt necessary to determine the effect of this drug on culture viability before studying its effects on oxygen consumption.

These data are listed in Tables IX - X.

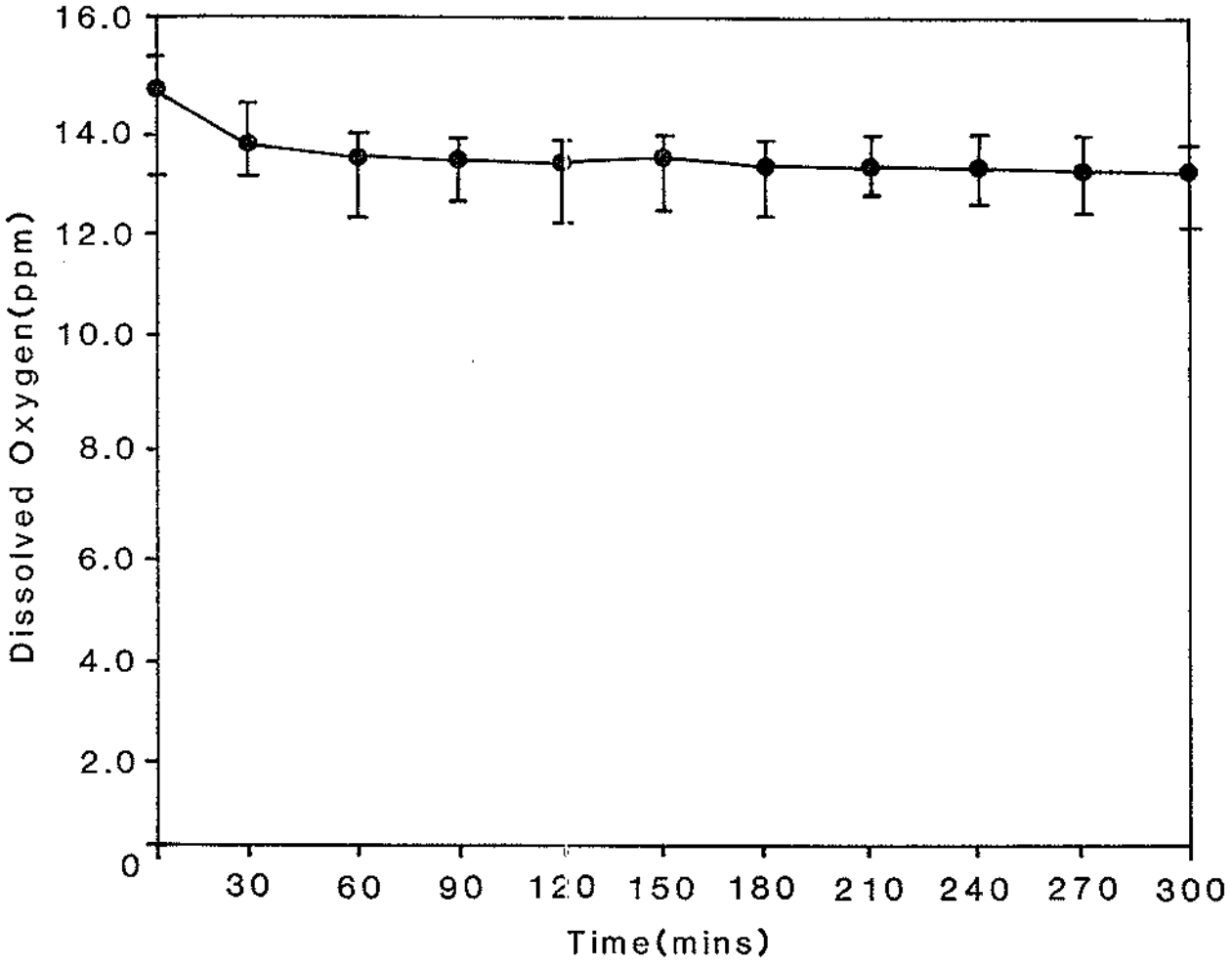
The survival of trophozoites after exposure to Quinacrine for 5 hours was determined since oxygen consumption assays were also carried out over a 5 hour period. (An assay defining the minimum lethal concentration (MLC) of Quinacrine in agarose over 5 days growth, served as a control). In semi-solid agarose medium, trophozoite colony forming efficiency (CFE) after 5 hours exposure to Quinacrine at concentrations up to 0.14 µg/ml in PBS; was maintained at 38-41%. At Quinacrine concentrations above 0.16 µg/ml, CFE decreased until a total loss of culture viability was observed at 2.0 µg/ml.

Since Quinacrine did not inhibit oxygen consumption at concentrations below MLC (See Fig 28); the effects of greater concentrations of Quinacrine on oxygen consumption were investigated. As a control, the influence of 1000 µg/ml Quinacrine on the D.O. level of sterile PBS was determined. (Fig 29).

Fig 30 illustrates the inhibition of oxygen consumption by Quinacrine dihydrochloride. At Quinacrine concentrations of 100-500 µg/ml there was little effect on oxygen consumption observed. Where trophozoites were exposed to 1000 µg/ml Quinacrine; oxygen consumption was limited to 50% of the levels observed in the absence of this inhibitor (see Fig 27).

All results expressed are the mean of 2-3 determinations.

Figure 26:Changes in the D.O. Level of Sterile PBS at 37°C.



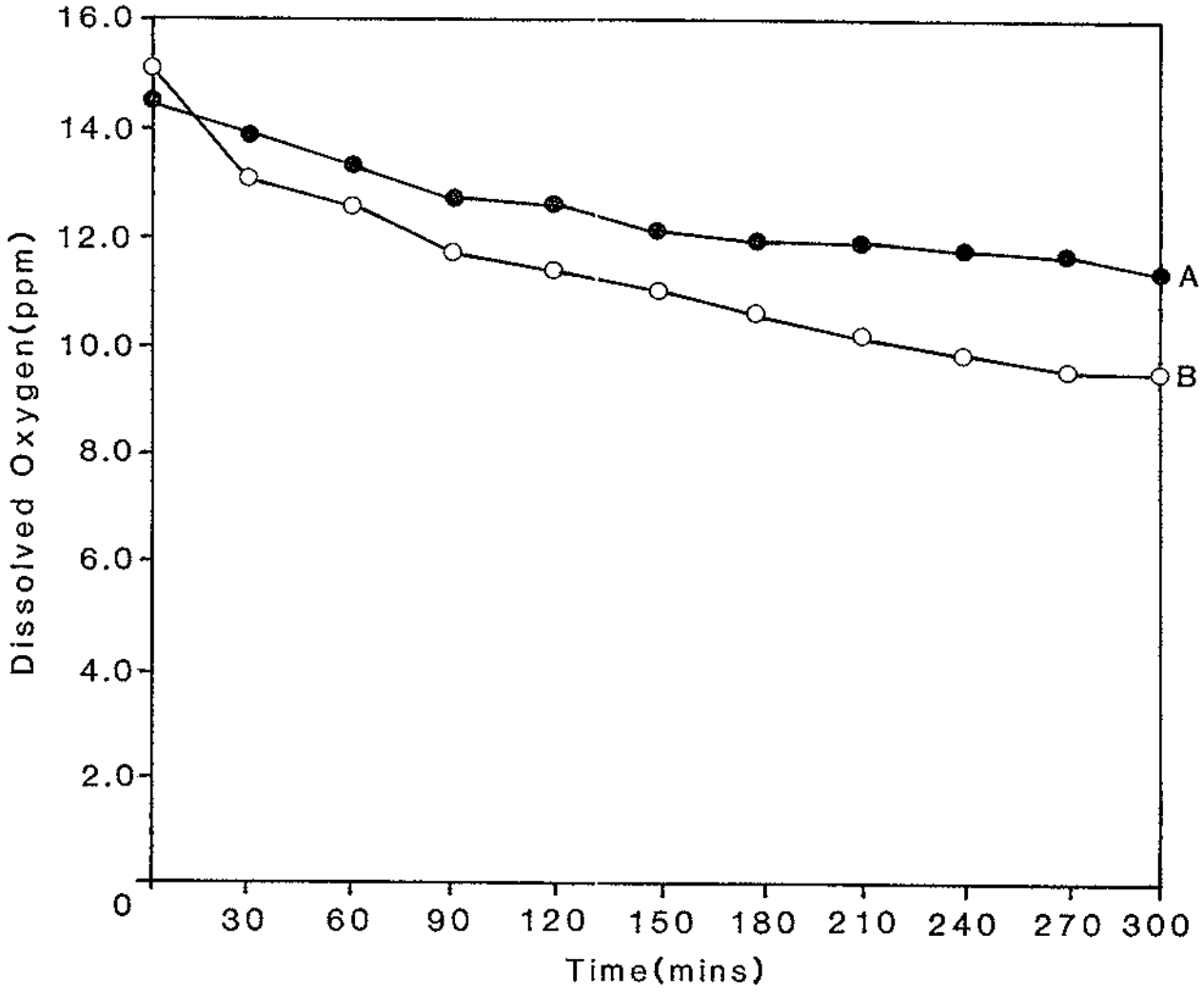
Total decrease in D.O. level of sterile PBS = 1.604 ppm/5 hours.

In Fig 27 the following legend applies:

● = A

○ = B

Figure 27: Oxygen Consumption by Trophozoite in PBS at 37°C.



Trophozoite suspension A:

Inoculum = 6.8×10^7 trophozoites
Total decrease in D.O. level =
3.409 ppm/5 hours
Oxygen Consumption/cell[a] =
5.3×10^{-9} ppm/cell/hr

Trophozoite suspension B:

Inoculum = 2.3×10^8 trophozoites
Total decrease in D.O. level =
5.833 ppm/5 hour
Oxygen consumption/cell =
3.7×10^{-9} ppm/cell/hr.

a. Oxygen Consumption/cell =

$$\frac{\text{Decrease in D.O. of suspension - decrease in D.O. sterile PBS}}{\text{Number of Trophozoites} \times \text{5 hours}}$$

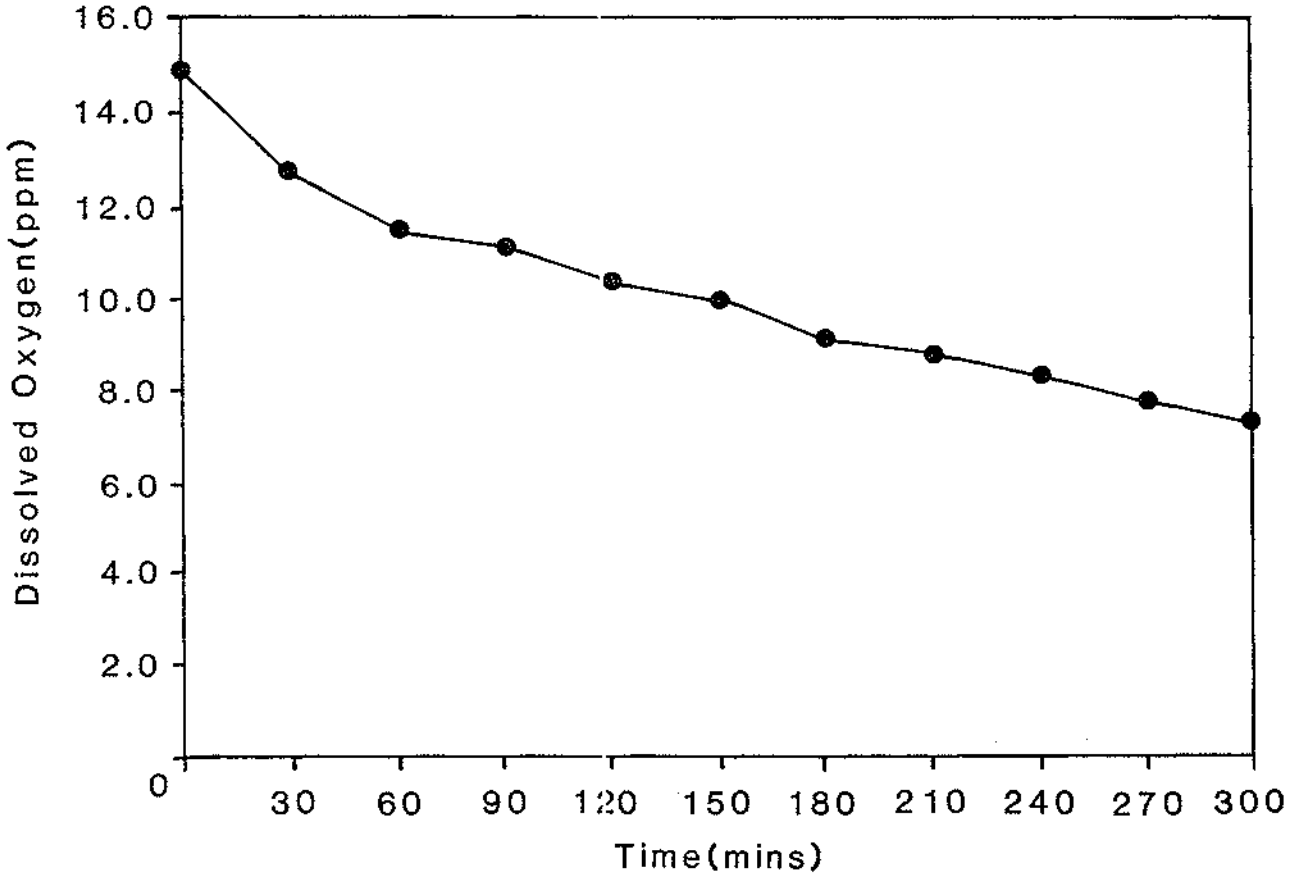
Table IX: The Viability of Trophozoites After Incubation in Semi-Solid Agarose Medium Supplemented with Quinacrine dihydrochloride.

Quinacrine Concentration (μg/ml)	No Trophozoite Colonies	CFE(%)
0.00	84	42
0.02	6	3
0.04	-	-
0.06	-	-
0.08	-	-
0.10	-	-
0.12	-	-
0.14	-	-
0.16	-	-
0.18	-	-
0.20	-	-

Table X: The Viability of Trophozoites After Five Hours Exposure to Quinacrine Dihydrochloride.

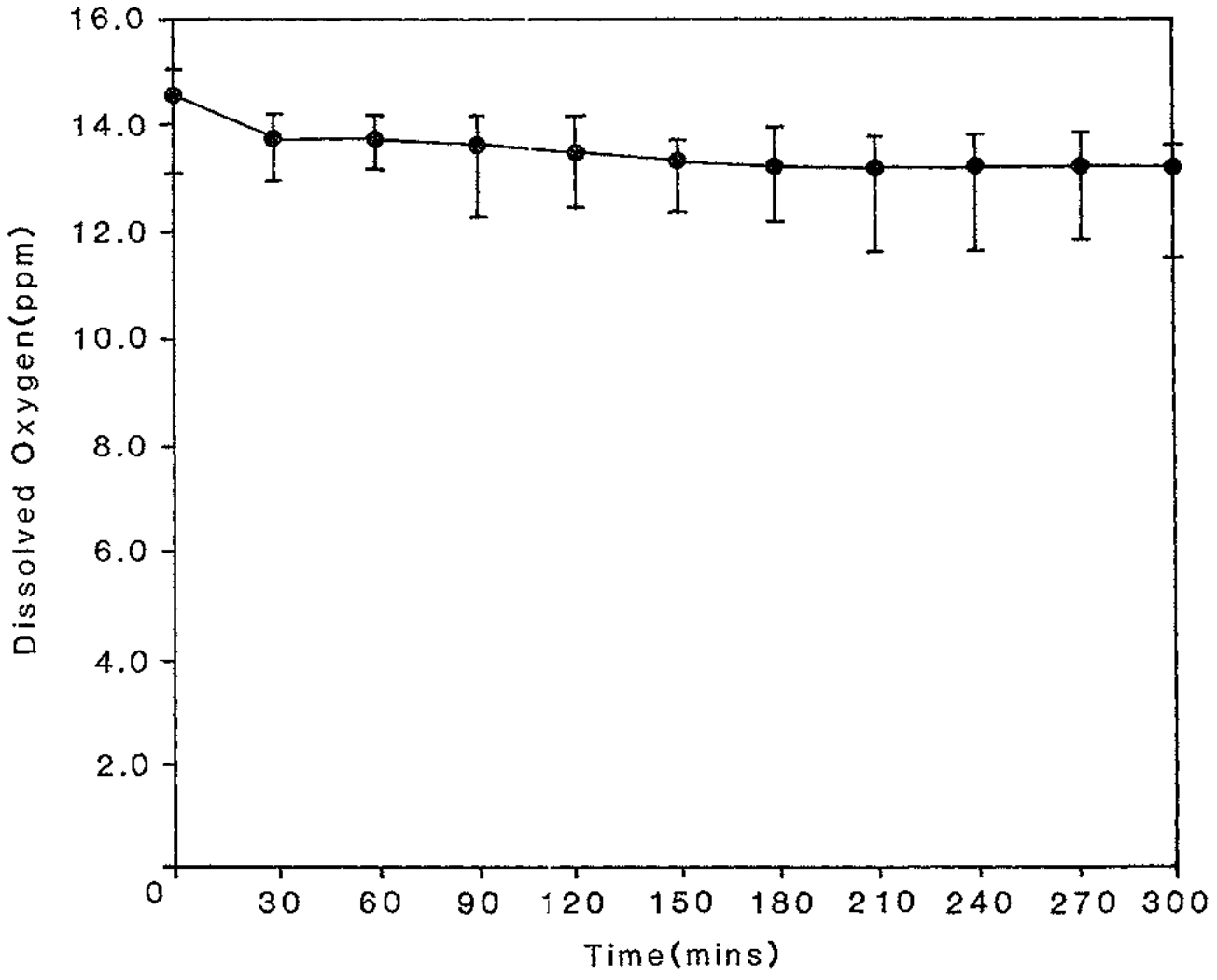
Quinacrine Concentration (μg/ml)	No Trophozoite Colonies	CFE(%)
0.00	76	38.0
0.02	79	39.5
0.04	82	41.0
0.06	77	38.5
0.08	76	38.0
0.1	80	40.0
0.12	79	39.5
0.14	79	39.5
0.16	75	37.5
0.18	63	31.5
0.2	60	30.0
0.5	38	19.0
1.0	25	12.5
2.0	-	-
3.0	-	-
4.0	-	-
5.0	-	-
6.0	-	-
7.0	-	-
8.0	-	-
9.0	-	-
10.0	-	-

Figure 28: The Effect of 1.0 μg/ml Quinacrine dihydrochloride on Oxygen Consumption By Trophozoites.



Inoculum = 2.4×10^{8} trophozoites
Total decrease in D.O. level = 6.649 ppm/5 hours
Oxygen consumption/cell = 4.2×10^{-9} ppm/cell/hr.

Figure 29: Changes in the D.O. Level of Sterile Quinacrine Dihydrochloride (1000 µg/ml) in PBS.

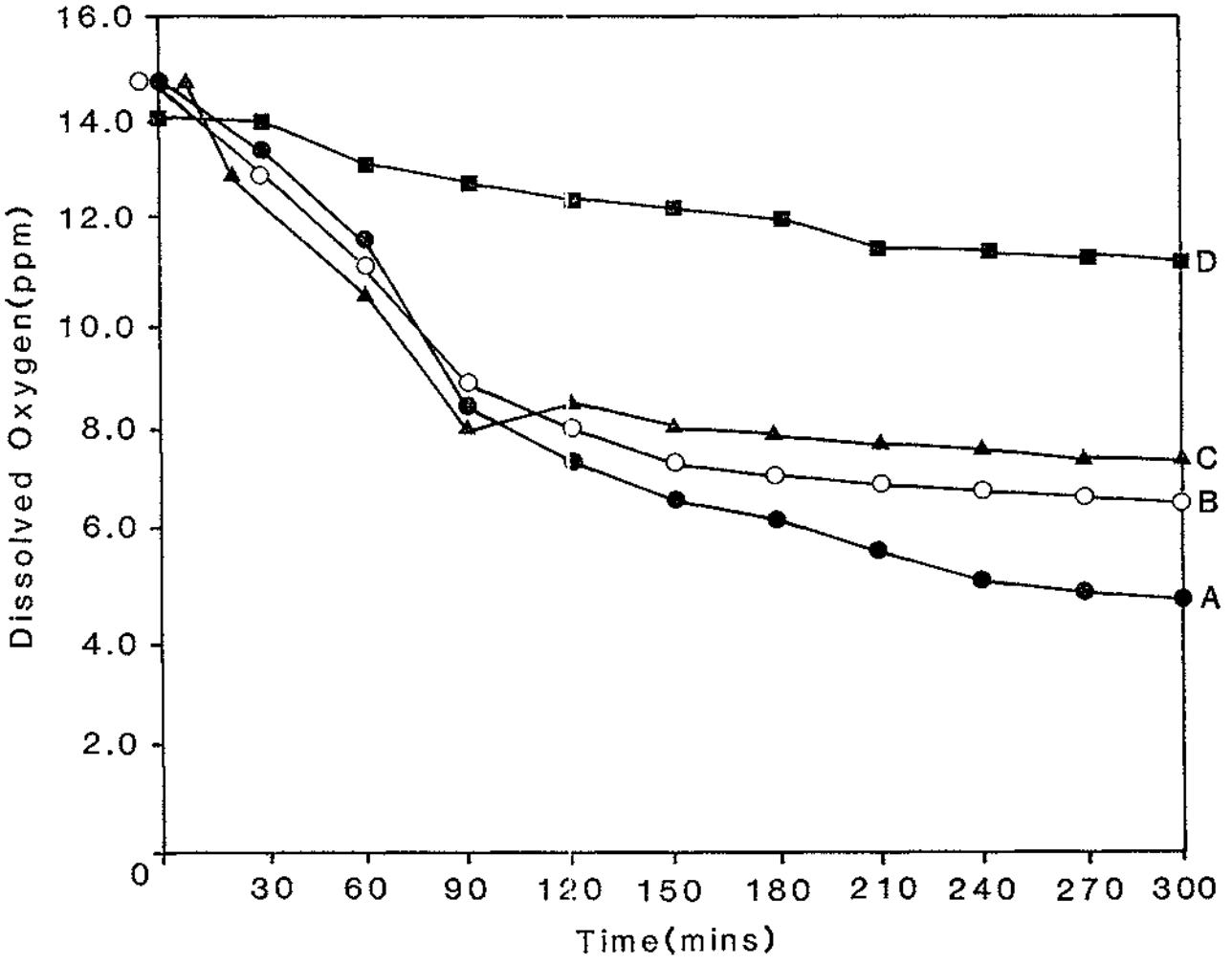


Total decrease in D.O. level of sterile Quinacrine dihydrochloride (1000 μg/ml) = 1.438 ppm/5 hours

In Figure 30 the following legend applies:

● = A
○ = B
▲ = C
■ = D

Figure 30: The Inhibition of Oxygen Consumption by Quinacrine Dihydrochloride.



Trophozoite Suspension A:

Inoculation = 2.5×10^8 trophozoites
Quinacrine dihydrochloride in PBS to a final conc of 100 µg/ml
Total decrease in D.O. level = 7.834 ppm/5 hours
Oxygen consumption/cell =
4.9×10^{-9} ppm/cell/hr

Trophozoite Suspension B:

Inoculum = 2.7×10^8 trophozoites
Quinacrine dihydrochloride in PBS to a final conc of 250 µg/ml
Total decrease in D.O. level = 6.255 ppm/5 hours
Oxygen consumption/cell =
3.4×10^{-9} ppm/cell/hr

Trophozoite Suspension C:

Inoculum = 2.4×10^8 trophozoites
Quinacrine dihydrochloride in PBS to a final conc of 500 µg/ml
Total decrease in D.O. level = 5.068 ppm/5 hours
Oxygen consumption/cell =
2.8×10^{-9} ppm/cell/hr

Trophozoite Suspension D:

Inoculum = 2.7×10^8 trophozoites
Quinacrine dihydrochloride in PBS to a final conc of 1000 µg/ml
Total decrease in D.O. level = 3.004 ppm/5 hours
Oxygen consumption/cell =
1.0×10^{-9} ppm/cell/hr

CHAPTER 5 : DISCUSSION

5.1 The Effect of pH and Temperature on Growth of Two Strains of G. intestinalis

Three distinct morphological groups within the genus Giardia have been defined by Filice (1952). Of these three groups; only G. intestinalis may be successfully cultivated in vitro. Axenic cultivation of this organism was first reported in 1976 by Meyer.

An increasing number of reports concerning the in vitro cultivation of G. intestinalis have outlined strict requirements for the optimal growth of axenic cultures.

Survival of G. intestinalis trophozoites is strongly dependant on the osmolality of growth media. Gillin and Reiner (1982), exposed trophozoites to NaCl and KCl in simple maintenance medium (MM-2) and complex growth medium (TPS-1). Trophozoites were tolerant to these salts at 200-300 mosmol/kg in MM-2, and 300-360 mosmol/kg in TPS-1. At NaCl/KCl concentrations below or above these limited ranges, both trophozoite attachment and survival were severely impaired.

To obtain optimal growth of cultures in TY1-S-33 growth medium, Keister (1983), found it necessary to supplement media with liquid or dehydrated bovine or porcine bile at a concentration of 50-100 mg/100 ml media. Farthing et al., (1983) also noted the importance of dehydrated bile in promoting culture growth in TY1-S-33. By supplementing media with bile at a concentration of 0.8 g/l; a culture generation time of 7.5 hours; the shortest generation time reported in the literature; was obtained. It was also observed that; although bile was stimulatory to growth; in excess; the presence of deconjugated bile salts may serve to disrupt trophozoites. (Farthing et al., 1983). There have been no further investigations reported concerning bile-promoted culture growth.

Even in complete and supplemented growth medium, efforts to establish axenic cultures may not be successful. In complex growth media such as TY1-S-33 and TPS-1, undefined biological products such

as trypticase soy broth and Parmede may vary from batch to batch in their ability to support culture growth. (Kasprzak and Majewska, 1985).

G. intestinalis trophozoites also exhibit strict requirements for L-cysteine, a reduced O-R potential, and reduced oxygen tensions for optimal growth. These points will be discussed later.

In the present study, the growth rates of two G. intestinalis strains; Bris/83/HEPU/106, and Hast/87/MUGU/68 in TY1-S-33 under control conditions (pH 7.15; temperature 37^{o}C); were recorded and then compared to growth rates of these same two strains when exposed to a range of non-optimal temperatures and pH levels. Those environmental factors not under investigation were consistent for all cultures examined; ie in all cases, 1-5 x 10^{5} trophozoites were incubated in complete TY1-S-33 in 14 ml screw-top culture tubes. Cultures were exposed to a range of pH levels at 37^{o}C; and similarly; cultures were exposed to a range of temperatures in TY1-S-33 at pH 7.15. Under these control conditions the growth rates of Bris/83/HEPU/106 and Hast/87/MUGU/68 were 0.085 gen/hr and 0.094 gen/hr respectively, equivalent to generation times of 11.8 and 10.8 hours respectively. (Figs 2 and 3).

The patterns of G. intestinalis growth in TY1-S-33 have been investigated by many workers. In most cases, recorded generation times are similar to those observed in this laboratory. Belosovic et al., (1982) recorded generation times of 9.66 hours in autoclaved TY1-S-33; and 7.69 hours in filtered TY1-S-33. From these figures he concluded that membrane filtration was the preferable method for the sterilization of TY1-S-33, and suggested a depletion of media components due to autoclaving. Kasprzak and Majewska (1985) cultured 20 G. intestinalis strains in TY1-S-33 and noted generation times for these cultures of 8.1-16.6 hours. In bile-supplemented TY1-S-33; Farthing et al., (1983) recorded a mean generation time of 7.5 hours. Keisteir (1983) also recorded very short generation times for strains of G. intestinalis; the minimum generation times observed being 6-7 hours.

The effect of pH and temperature on these growth rates has not been reported. Since the advent of axenic cultivation, incubation of cultures has been at physiological temperatures in a range of complex growth media: 35.5°C in TY1-S-33 (Gillin and Diamond, 1981a); 36°C in TY1-S-33 (Keister, 1983); 37°C in TPS-1 (Visvesvara, 1980; Farthing et al., 1982; Hill et al., 1986); and 37°C in TY1-S-33 (Weider et al., 1983; Kasprzak and Majewska, 1985). Gillin and Reiner, (1982) investigated the effect of incubation temperature on the attachment of G. intestinalis trophozoites in vitro. Using a simple maintenance medium (MM-2); these workers observed a reduced rate of attachment at 24°C, and a complete inhibition of attachment below 12°C. In addition, the pH optima for trophozoite attachment were defined as being within the range pH 6.85-7.0.

Trophozoite attachment levels in vitro can be used as an indication of the general vigour of cultures. (Gillin and Reiner (1982). Since it has been demonstrated that reduced temperatures adversely effect trophozoite attachment (Gillin and Reiner, 1982); it was not surprising to record a similar correlation between temperature and growth rates of the two strains examined. Clearly defined optimal temperature ranges for culture growth were demonstrated in this study (Figs 4 and 5; Tables XI and XII). Cultures exposed to temperatures more than 3°C above or below physiological temperature, grew at markedly reduced rates. From these results it can be concluded that G. intestinalis growth and survival is only possible over a very limited temperature range. Cultures declined at 30°C, and rapid culture death was observed at 45°C (See Appendix). The ability of G. intestinalis to adapt to growth at non-optimal temperatures was not investigated.

A similarly restricted pH range for culture growth and survival was observed in G. intestinalis. Hast/87/MUGU/68 trophozoites were less tolerant than Bris/83/HEPU/106 to pH levels above 7.25 (Figs 6 and 7; Tables XIII and XIV). The growth of both Bris/83/HEPU/106 and Hast/87/MUGU/68 were significantly inhibited by acid pH. This study demonstrated the optimal growth of Bris/83/HEPU/106 and Hast/87/MUGU/68 in medium under neutral pH conditions (6.75-7.5).

Under these conditions, growth of cultures occurred at rates similar to those observed under the control conditions described earlier, ie:

Bris/83/HEPU/106:	pH 7.0-7.5	:	growth rate = 0.08-0.088 gen/hr
	pH 7.15 (control)	:	growth rate = 0.085 gen/hr
Hast/87/MUGU/68:	pH 6.75-7.25	:	growth rate = 0.085-0.105 gen/hr
	pH 7.15 (control)	:	growth rate = 0.093 gen/hr

These results are consistent with the reported successful growth of axenic cultures in complex media adjusted to near neutral pH. The first axenic G. intestinalis cultures were established by Meyer (1976), in an HSP-1 medium which was maintained at pH 6.8-7.0. Complex growth media specifically developed for axenic cultivation of G. intestinalis; TPS-1 (Visvesvara, 1980), and TY1-S-33 (Diamond et al., 1978) are utilised after adjustment to pH levels of 6.8 and 7.0-7.2 respectively.

Generally, the adverse influence upon cultures, of non-optimal conditions such as those investigated during this study; may be attributed to three possible effects:

i. All cells in culture multiply more slowly
ii. Fewer cells multiply, but a normal rate
iii. Fewer cells multiply at a reduced rate. (Gillin and Diamond, 1980).

The observed growth patterns of cultures under non-optimal pH and temperature indicate that not all cells in culture continue to divide; in fact, under elevated temperature (45^{o}C); rapid culture death occurred. Therefore, any decrease in growth rate observed in this study was more likely to be due to a decrease in the number of dividing cells, rather than an actual change in generation time.

In vivo G. intestinalis colonises the duodenum of the infected host. Trophozoites may also be isolated from the gall bladder (Ament and Rubin, 1972). The in vivo environment of G. intestinalis has been used as a guideline in efforts to establish an ideal in vitro environment for culture growth; hence the use of physiological temperatures for the incubation of axenic cultures. Keister (1983)

developed bile supplemented TY1-S-33 for *in vitro* cultivation after observing the colonisation of the small intestine by *G. intestinalis* and interpreting this as a positive response to a bile-rich environment.

pH may also have a profound influence on the *in vivo* growth of *G. intestinalis*. (Kofoid *et al.*, (1933) demonstrated a direct correlation between the distribution of a number of intestinal protozoa; including *G. intestinalis*; in the gut of *Rattus norvegicus*; and the hydrogen ion concentration of the intestine wall. *G. intestinalis* trophozoites were observed 6-15 inches posterior to the stomach in the jejunum of the six rats examined. The average pH of the intestinal wall at this level was 6.45-6.52. During the current study, the growth of Bris/83/HEPU/106 and Hast/87/MUGU/68 over the pH range 6.25-6.5 occurred at a reduced rate compared to culture growth under control conditions. It must be noted, however, that direct comparison between an axenic *in vitro* growth response; and an *in vivo* growth response in the presence of a mixed intestinal flora is not feasible. Gillin and Reiner (1982) noted that 'local variations in pH may influence the attachment of trophozoites to the intestinal epithelium; and mediate the differentiation of motile trophozoites into the cyst form lower in the digestive trace'. *In vitro* excystation of *G. intestinalis* has also been found to be pH-dependant; occurring at a pH of 2.0. (Bingham and Meyer, 1979; Rice and Schaefer, 1981; Schaefer *et al.*, 1984; Feely, 1986; Isaac-Renton *et al.*, 1986; Kaur *et al.*, 1986).

5.2 Quantitative Clonal Growth of G.intestinalis in Semi-Solid Aganose Medium

Axenic cultivation of *G. intestinalis* is possible in semi-solid media. Often these semi-solid media are developed after agar-modification of standard liquid growth media. An agar-modified medium (M3 + 1-1.5% agar) was utilised by Teras and Lakhonina (1975) (see Meyer and Radalescu, 1984) to establish *G. intestinalis* cultures after isolation of trophozoites from 13 rabbit sources.

Clonal growth of *Entamoeba histolytica* in an ionagar-supplemented

TY1-S-33 medium constitutes a useful method for quantifying the viability of cells (Gillin and Diamond, 1978). In 1980, Gillin and Diamond adapted this methodology to the growth of G. intestinalis in agarose medium, also based on TY1-S-33. Since the 'ability of a single cell to multiply and form a colony is a stringent criterion for viability'; such a method was said to be 'quantitative, sensitive and convenient' (Gillin and Diamond, 1980).

The aim of this study was to examine the growth of Bris/83/HEPU/106 and Hast/87/MUGU/68 in semi-solid agarose media and assess the value of clonal growth as a quantitative, reproducible assay of trophozoites in this laboratory.

Following the methodology outlined by Gillin and Diamond (1980); trophozoites were incubated in media representing a range of final agarose concentrations (0.18-0.25% w/v). Inoculated medium was allowed to solidify either at room temperature, or in an ice-water bath for 10 minutes. After cultures were incubated at 37^{o}C for 5-7 days colony forming efficiency (CFE %) was calculated according to Gillin and Diamond (1980), ie 'CFE = the number of colonies growen, divided by the number of cells inoculated, multiplied by 100.'

Gillin and Diamond (1980) reported a CFE of 20-40%. CFE was said to be dependant upon both agarose concentration and the method of cooling. Optimal conditions for the growth of G. intestinalis in semi-solid agarose medium were defined as follows: 0.17% w/v agarose cooled rapidly in ice; or; 0.22% agarose allowed to cool slowly at room temperature.

Tables II and III demonstrate the inconsistent nature of G. intestinalis CFE obtained during this study. Despite duplication of each assay, the growth of G. intestinalis in agarose at all concentrations remained unpredictable. Bris/83/HEPU/106 cultures grew at 0.18% agarose under both cooling conditions, but resultant colony forming efficiency was variable (11-32%). In some cases, growth of cultures did not occur at all. The uneven distribution of colonies within the ager matrix hindered attempts to accurately calculate the CFE of cultures. Often, the accumulation of colonies

at the base of culture tubes made enumeration of colonies impractcable. The colony forming efficiency of Hast/87/MUGU/68 in all agarose concentrations examined was high (40-60%), but; again; growth of cultures was unpredictable and varied greatly. For these reasons, it was concluded that unless some degree of consistency in CFE could be ensured, it was not practical to base estimates of culture viability after exposure to unfavourable experimental conditions (see later discussion), upon the clonal growth of cultures in agarose.

Gillin and Diamond (1980) did not comment on the extent of variability in CFE observed during their study; but the reported CFE (20-40%) indicates that such variability did, in fact, occur. These workers also noted that a higher CFE was obtained more consistently with formerly attached than with free cells. (In accordance with this finding, all agarose assays carried out during the course of this study were initiated with formerly attached cells). While investigating the growth of E. histolytica in ionagar medium; Gillin and Diamond (1978) observed a correlation between reductions in CFE, and an increase in the age of cultures used to inoculate agar medium. It would seem reasonable to assume a similar correlation exists for clonal growth of G. intestinalis in agarose medium.

The variability of CFE observed during this study may be due to several factors. The ability of E. histolytica trophozoites to 'escape' from the agar matrix and grow on the glass walls of culture tubes has been demonstrated (Gillin and Diamond, 1978). Similar movement by G. intestinalis trophozoites would extensively reduce the number of colonies observed in culture tubes during this study.

A further cause of reduced CFE may be the methodology itself. The viability of trophozoites may be adversely effected by exposure to temperature variation. (Gillin and Diamond, 1981) (During the course of agarose culture establishment; trophozoites are removed from 37^{o}C incubation; chilled during detachment from culture vessels; inoculated into TY1-S-33 at 42-45^{o}C which has been supplemented with molten agarose maintained at 65^{o}C). Such a proposal would be consistent with the observation that during

preliminary studies; if the inoculation of trophozoites too rapidly followed the addition of agarose; culture growth did not occur.

Once established, G. intestinalis colonies can be removed from agar medium, and used to re-establish axenic culture in liquid medium. Colonies suspended in an agar matrix may be viable for an extended period since the oxidation of L-cysteine (a component of TY1-S-33) in semi-solid medium is likely to be slower due to a retarded diffusion of oxygen (Gillin and Diamond, 1981). While the clonal growth of G. intestinalis in agarose may have only limited value as an assay of trophozoite viability in some instances; the growth of discrete colonies in agarose does allow the isolation of clones, ie 'groups of cells that are the progeny of single cells' (Gillin and Diamond, 1980). In agarose; the probability that any isolate has arisen from a single trophozoite is 90%. By repeating the procedure, this probability increases to 99% (Gillin and Diamond, 1980). The routine isolation of G. intestinalis clones may enable investigators to carry out a number of detailed studies concerning the metabolism and genetic make-up of G. intestinalis. It is in such fields that quantitative, reproducible clonal growth of G. intestinalis must be available.

5.3 The Effect of Redox Potential and L-cysteine Concentration on Culture Growth and Trophozoite Attachment in vitro

5.3.1 Culture Growth

As noted earlier (4.3; 5.1), G. intestinalis is cultivated in complex media under reducing conditions which are maintained with the addition of two reducing agents to TY1-S-33; L-cysteine (0.15% w/v) and L-Ascorbic acid (0.02% w/v). Oxidation of cysteine (a thiol reducing agent) is rapid at physiological pH and temperature, and enhanced in the presence of iron and other metal complexes which are effective catalysts at pH 7.0-8.0. Thiols do not auto-oxidise without a catalyst, and rates of oxidation are proportional to catalyst concentration (Jocelyn, 1972).

L-cysteine is known to be an essential component of TY1-S-33, though the functions of this thiol reducing agent in complex growth media have not been determined. Rapid growth of G. intestinalis subsequent to supplementation of TPS-1 with 0.2% w/v L-cysteine has been reported. (Kasprzak and Majewska, 1983).

Gillin and Diamond (1981a) demonstrated that complex growth media (TY1-S-33 and TPS-1) lacking L-cysteine were unable to support growth of G. intestinalis. A specific requirement for L-cysteine was especially marked in TY1-S-33, as cultures grown in media which included alternative reducing agents yielded only 7-33% of the optimal growth observed in the presence of L-cysteine.

From of trophozoite numbers in experimental cultures after 96 hours incubation, optimal growth of these cultures was recorded in TY1-S-33 supplemented with L-cysteine at 6-24 mM concentration (Gillin and Diamond, 1981a).

In view of the paucity of information available regarding the general physiology of G. intestinalis growth in culture; the efficacies of a range of L-cysteine concentrations (% w/v), and reducing conditions (O-R potential in mV) in supporting growth and attachment of this organism, were determined.

According to the protocol described earlier (See 3.4.1) L-cysteine was incorporated in different amounts in TY1-S-33 to create a range of negative O-R potentials. Growth of both G. intestinalis strains examined (Bris/83/HEPU/106 and Hast/87/MUGU/68), was strongly dependant upon L-cysteine concentration and O-R potential. A direct correlation existed between the increasing L-cysteine concentration/decreasing O-R potential of growth media; and increasing growth rates of experimental cultures. (Figs 8 and 9; Tables XV and XVI).

Reducing conditions are essential for optimal growth of G. intestinalis (Meyer, 1976); however; it must be noted that;

while O-R potentials in TY1-S-33 become less negative where L-cysteine concentrations are reduced; the corresponding decreases in the growth rates of cultures in this study cannot be attributable solely to these changes in O-R potential. Gillin and Diamond (1981a) have indicated that; even under ideal reducing conditions; the growth of G. intestinalis is impaired in the absence of L-cysteine, though the specific metabolic function of L-cysteine has not yet been determined.

From this it may be concluded that the growth of cultures exposed to both elevated redox potentials and reduced L-cysteine concentrations would be markedly reduced. A reduction in the growth rate of cultures exposed to these conditions (0.00-0.10% L-cysteine and -114.7 to -243.6 mV) was recorded in this study (Figs 8 and 9; Tables XV and XVI); with rapid culture death occurring in the absence of L-cysteine. In media containing L-cysteine at concentrations above 0.15% w/v (-272.4 to -302.1 mV) a maximal rate of culture growth was achieved. In accordance with the findings of Gillin and Diamond (1981a); L-cysteine at a concentration of 6 mM (0.10% w/v) supported culture growth at 94-99% the maximal rate (Tables XV and XVI). The inhibitory effect upon culture growth of L-cysteine at concentrations above 24 mM (Gillin and Diamond, 1981a) was not investigated.

5.3.2 Trophozoite Attachment in vitro

When axenically cultivated in vitro; G. intestinalis trophozoites exhibit a striking tendency to attach to the walls of a culture vessel to produce a confluent monolayer. During the log phase of culture growth, trophozoites may detach and reattach spontaneously. (Erlandsen and Feely, 1984).

Gillin and Diamond (1981a,b) have outlined the kinetics of trophozoite attachment in both complex media (TPS-1) and simple maintenance media (MM-2). Attachment of trophozoites is most rapid in the first two hours following culture establishment. At this stage 85-90% of trophozoites may be attached to the

culture vessel under ideal conditions. This ratio of cell attachment is comparable to the observed attachment of Bris/83/HEPU/106 and Hast/87/MUGU/68 in complete TY1-S-33. (Figs 10 and 11).

Trophozoites attached to culture tube surfaces rapidly over a 60-120 minute period, to a final trophozoite attachment level of 84-86%.

A number of environmental factors have been shown to significantly influence attachment of G. intestinalis trophozoites in vitro. The crude Cohn III fraction of bovine serum (0.05 µg/ml) is stimulatory to trophozoite attachment in MM-2 medium. Attached trophozoite numbers increase with elevated Cohn III concentrations (Gillin and Reiner, 1982).

Zenian and Gillin (1984) have noted the attachment and survival-promoting activities of a low density, protein-rich fraction within human and rabbit duodenal mucus, which stimulates trophozoite attachment at a concentration of 1-1000 µg/ml in Eagles Minimal Medium (H-MEM). At concentrations above 1000 µg/ml, duodenal mucus is inhibitory to trophozoite attachment.

Maximal attachment of trophozoites in axenic culture is obtained only under a rigid set of environmental conditions. In this context, the effect of pH and temperature upon trophozoite attachment have already been discussed (5.1). Attachment of G. intestinalis in culture is also strongly dependant on the presence of reducing agents (Gillin and Diamond, 1981b). While G. intestinalis exhibits a specific L-cysteine requirement for culture growth (Gillin and Diamond, 1981a); the thiol requirement for trophozoite attachment is less specific. A number of thiol reducing agents (D- cysteine; thiomalic acid; α-mercaptopropionyl glycine; glutathione and N-acetyl cysteine) support trophozoite attachment at 63-84% of that observed in L-cysteine-supplemented medium. (All reducing agents at a concentration of 6 mM). (Gillin and Reiner, 1982).

L-Ascorbic acid is stimulatory to trophozoite attachment in the presence of thiols; but is ineffective alone (Gillin and Diamond, 1981b; Gillin and Reiner, 1982).

In continuation of this work; Figs 12 and 13; Tables IV and V, demonstrate a relationship between L-cysteine concentration (% w/v) in TY1-S-33 and attachment of G. intestinalis in culture With increasing L-cysteine concentrations, and corresponding decreases in O-R potential; Bris/83/HEPU/106 and Hast/87/MUGU/68 trophozoites exhibited an increasing tendency to attach in culture; to a maximum trophozoite attachment level of 90-96%. Trophozoite attachment levels were reduced to less than 50% in the absence of L-cysteine. The observed loss of trophozoite ability to attach may be an early step in the eventual loss of viability caused by L-cysteine deprivation (Gillin and Diamond, 1981b). This would be in accordance with the idea that trophozoite attachment is an indication of the general vigour of cultures (5.1).

From these results it may be concluded that maximal culture growth and attachment of G. intestinalis are obtained in TY1-S-33 under the following conditions; L-cysteine concentration: 0.10-0.15% w/v; OR potential: -243.6 to -272.4 mV); and that under more extreme reducing conditions the growth rate of cultures; and the level of trophozoite attachment only increase by minimal increments.

A close correlation between the redox potential of growth media, and culture growth has been recorded for E. histolytica; an aerotolerant anaerobe which displays a cell physiology similar to G. intestinalis (Gillin and Diamond, 1980, 1981a,b). Longevity of cultures is associated with low O-R ptoentials, with most prolific culture growth at -300 to -500 mV. (Jacobs, 1941; Chang, 1946; Singh et al., 1973, 1974). In vitro; sharp increases in O-R potential after oxygenation, induce mass encystment of E. histolytica trophozoites (Chang, 1946). In vivo; local variations in O-R potential may effect the attachment of E. histolytica to host intestinal epithelium, and

influence encystment (Gillin and Reiner, 1982).

Reducing agents are necessary for axenic growth of G. intestinalis; and L-cysteine in growth medium functions to reduce O-R potentials. Gillin and Diamond (1981b) have reported, both, the ability of L-cysteine to protect trophozoites from the lethal effects of high D.O. levels in growth medium (see 4.4; 5.4); and; the maintenance of reduced O_2-tensions in culture due to the presence of L-cysteine with L-Ascorbic acid in TPS-1 and TYI-S-33. However; L-cysteine has importance beyond its function as a reducing agent, since it is required for trophozoite attachment in culture, even under N_2 atmospheres (Gillin and Reiner, 1982).

The requirements for L-cysteine to promote trophozoite attachment and culture growth are more specific than the function of cysteine as a reducing agent (Gillin and Diamond, 1981b) ie L-cysteine in growth medium meets specific metabolic requirements, while the O-R potential of culture may be effectively reduced by a number of reducing agents.

It seems most likely that L-cysteine is utilised by G. intestinalis in its oxidised form. As mentioned earlier; oxidation of L-cysteine is rapid at physiological pH and temperature, and in the presence of iron (Jocelyn, 1972). Since these conditions apply in the complex growth media used for axenic cultivation of G. intestinalis, the observation by Gillin and Diamond (1981b); (using the Ellman reaction for calorimetric analysis); that 20% of the L-cysteine added to growth media is oxidised within the first three hours of incubation; is not surprising. Under the mild oxidising conditions of culture, the major products of L-cysteine oxidation are disulphides. Gillin and Diamond (1981b) have suggested that mixed disulphides formed between oxidised L-cysteine and peptides in TYI-S-33 may be utilised by G. intestinalis. Once cysteine is oxidised in complex growth medium, such mixed disulphides would predominate over cystine due to their increased solubility.

Eagle (1960) has indicated that the -SH groups of human serum protein are able to react with cystine, to bind half-cystine residues in stable disulphide linkages. Alternatively; cysteine may react directly with protein-SH groups in an oxidative reaction to produce the same disulphide linkage. Both these reactions could be catalysed by iron in TY1-S-33.

While the question of disulphide utilisation by G. intestinalis has not yet been resolved; it is known that trophozoites require high concentrations of exogenous L-cysteine for culture growth. The inability of cultures to grow in autoclaved TY1-S-33, has been attributed to a decrease in exogenous L-cysteine levels subsequent to autoclaving. (Belosovic et al., 1982).

G. intestinalis is able to concentrate ^{35}S-cysteine from growth medium (Gillin and Diamond, 1981b). It is not known if trophozoites are able to synthesise L-cysteine; though the presence of a disulphide reductase in this organism has been proposed (Gillin and Diamond, 1981b). Iron present in TY1-S-33 is an important catalyst for oxidation of L-cysteine (See earlier discussion). This iron may form complexes with cysteinyl-SH groups within proteins to produce iron-sulphur proteins (Neilands, 1974; Gillin and Diamond, 1981b). Since Fe-S centres are an essential component of the electron-transport system of G. intestinalis (See 5.5); the specific requirement of G. intestinalis for L-cysteine to support growth and attachment in culture, may be attributable to this iron-cysteine complexing in growth medium. (Gillin and Diamond, 1981a).

5.4 Sensitivity of G. intestinalis Trophozoites to Elevated D.O. Levels in TY1-S-33

Although optimal growth of G. intestinalis in culture occurs only under reduced oxygen tensions in growth medium (5.1; 5.3); trophozoites are aerotolerant to a limited degree (Gillin and Diamond, 1981b).

These workers measured the oxygen-sensitivity of trophozoites by exposing cultures to elevated oxygen tensions which they adjusted by varying the volume of growth medium in 4 ml culture-tubes. 'High PO_2' levels (130-144 mmHg or 19-20% O_2); were obtained in culture tubes containing medium in 0.5 ml aliquots. This method did not allow for a fine control of the PO_2 throughout the experimental period.

In the present study, trophozoites were directly exposed to pure oxygen which was bubbled through TY1-S-33 growth medium in 1 l culture vessels (See 3.5.2). (NB: The D.O. level of freshly prepared TY1-S-33 at 37°C in this laboratory was 0.4-0.8 ppm). The exposure of trophozoites to oxygen was directly controlled through the constant adjustment of oxygen flow-rates.

Direct exposure of G. intestinalis to a precisely determined D.O. level in growth medium represents a sensitive, reproducible method of determiningg the oxygen-sensitivity of trophozoites in vitro.

Under the experimental culture conditions described; elevation of D.O. levels (ppm) in TY1-S-33 at 37°C resulted in decreases in the $T_{½}$ killing of the two strains examined; Bris/83/HEPU/106 and Hast/87/MUGU/68. (See Tables VI and VII). This was not unexpected since an earlier report had indicated that under increased oxygen tensions in growth media, exponential killing of cultures occurs regardless of the presence of reducing agents; although, when present, L-cysteine and Ascorbic acid delayed the onset of this killing by up to 11 hours under 'high PO_2' conditions. (136-144 mmHg). (Gillin and Diamond, 1981b).

It must be noted; however; that direct comparisons should not be made between the oxygen sensitivity of Bris/83/HEPU/106 and Hast/87/MUGU/68 recorded during the course of this study; and the oxygen sensitivity of G. intestinalis observed by Gillin and Diamond (1981b); whose methods are considered to be less controlled and accurate than those used in this study.

Bris/83/HEPU/106 trophozoites were then exposed to elevated D.O.

levels (8.0-12.0 ppm) at reduced temperatures. Reduced sensitivity to elevated oxygen tensions where temperatures are below those used for routine incubation of cultures, has been previously demonstrated in Entamoeba histolytica and Entamoeba invadens (Gillin and Diamond, 1980). Several similarities exist between G. intestinalis and E. histolytica even though the two intestinal protozoa are unrelated. (Gillin and Diamond, 1981b). For this reason, the behaviour of E. histolytica is often used as an indication of behaviour that may be expected from G. intestinalis under the same experimental conditions. This point will be returned to later.

From Figs 23-25 and Table VIII; it is interesting to note that Bris/83/HEPU/106 also displayed a degree of 'temperature-dependant oxygen sensitivity'. At reduced temperatures ($20^{o}C$ - $30^{o}C$); D.O. levels which had earlier caused rapid culture death at $37^{o}C$; were less lethal to trophozoites ie both the $T_{\frac{1}{2}}$ killing of cultures; and the lag phases preceding exponential killing were extended. (See Figs 23-25).

The reasons for oxygen-sensitivity of trophozoites may be manyfold and are not completely understood. It is not known if the death of cultures upon exposure to elevated D.O. levels is a direct effect of oxygen itself, or is due to oxygen products (Gillin and Diamond, 1980). Univalent reduction of oxygen produces a number of products which can be toxic to most forms of life. (Fridovich, 1977).

The following reactions illustrate the univalent pathway of oxygen reduction:

(a) $O_2 + e^- = O_2^-$ (superoxide anion radical)

(b) $O_2 + e^- + 2H^+ = H_2O_2$ (hydrogen peroxide)

(c) $H_2O_2 + e^- + H^+ = H_2O + OH.$ (hydroxyl radical)

(d) $OH. + e^- + H^+ = H_2O.$

It is possible that these products of oxygen reduction in TY1-S-33 growth medium may accumulate in excess of the ability of G. intestinalis trophozoites to deal with them, as has been postulated for E. histolytica (Band and Cirrito, 1979).

G. intestinalis may possess some form of active defense against oxygen radicals, for example superoxide dismutase activity (2400 ± 500 mU/mg protein) has been detected in trophozoites (Lindmark, 1980); and could serve to catalyse the dismutation of superoxide radicals via an oxidation-reduction reaction.

$$O_2^- + O_2^- + 2H^+ = H_2O_2 + O_2$$

Thus superoxide dismutase enzymes in G. intestinalis may be essential protection against toxic species of oxygen (Fridovich, 1977).

Oxygen-sensitive G. intestinalis trophozoites have been shown to actively consume oxygen (see 5.5). Similar activities observed in E. histolytica have led to suggestions that this oxygen consumption represents some form of 'O_2 scavenging' as a protective mechanism against toxic oxygen radicals which may accumulate under reducing conditions in TYI-S-33 (Gillin and Diamond, 1980). But the observed reduction in sensitivity of E. histolytica trophozoites to oxygen at 0^oC where cells were assumed to be metabolically inactive did not support the idea of detoxification of oxygen radicals by oxygen consumption (Gillin and Diamond, 1980).

Similarly in this study, the reduced sensitivity of G. intestinalis trophozoites to oxygen at temperatures where cell metabolism is assumed to be less active, indicates that culture survival under elevated D.O. levels should not therefore, be solely attributed to a protective effect exerted in the consumption of oxygen by G. intestinalis during carbohydrate metabolism (See 5.5).

The bizarre division forms and 'clumped' trophozoites observed under increased oxygen tensions in this study (See Plates 4-5); have been described by a number of workers (Gillin and Diamond, 1981; Boreham et al., 1984). Inability of trophozoites to divide successfully and the clumping of deformed trophozoites is obviously a function of the death of cultures under these conditions.

Dissolved oxygen in growth medium may have a direct effect on the ultrastructure of trophozoites. For example singlet oxygen;

produced through the 'excitation' of ground-state oxygen; is a powerful oxidant, which attacks polyunsaturated fatty acids. (Fridovich, 1977). Such fatty acids are plentiful in cell and organelle membranes. (Davis et al., 1980; Taylor and Cannalier, 1982). Lipid peroxidation by singlet oxygen (Fridovich, 1977); would severely damage trophozoite cell membranes if it was to occur in cultures under elevated D.O. conditions.

5.5 Oxygen Consumption by G.intestinalis Trophozoites

The basis for the assumption that G. intestinalis is an anaerobe, has, in the past; been that the normal environment of this protozoan, (the gut) is essentially anaerobic (Moreckei and Parker, 1967; Owen et al., 1979), and; that a low redox potential is required for its optimal growth in culture (5.3; 5.4). Furthermore; G. intestinalis trophozoites lack any structures identifiable as mitochondria (Friend, 1966). However; during the course of this study; both trophozoite aerotolerance (see 5.4); and oxygen consumption by trophozoites have been demonstrated.

Utilisation of oxygen was investigated according to the protocol described earlier (3.6). 'High-yield' Hast/87/MUGU/68 suspensions (6.8×10^7 - 2.6×10^8 cells) were incubated in PBS solution. Oxygen consumed by these trophozoites was measured in terms of D.O. changes in the suspension as a function of time. All decreases in the D.O. level in excess of those observed in controls (sterile PBS at 37^oC); were due to consumption of dissolved oxygen by trophozoites. Dissolved oxygen was removed from PBS solution by trophozoites at a rate of $3.7 - 5.3 \times 10^{-9}$ ppm/cell/hr. (See Fig 27).

G. intestinalis displays a high affinity for oxygen when the latter is present in low concentrations (see 5.4); and rapid consumption of this gas has been documented by several investigators. Lindmark (1980); utilising microrespirometric techniques; recorded oxygen consumption by trophozoites at a rate of 93 ± 10 nmol O_2/min/mg protein. A similarly vigorous consumption of oxygen was demonstrated polarographically by Weinbach et al., (1980). In both

cases, consumption of oxygen by trophozoites was independant of oxygen concentration with Weinbach et al., (1980) noting a reduction in the rate of trophozoite respiration only as oxygen concentrations in suspension approached zero. This oxygen consumption was stimulated by glucose (33 mM) (Weinbach et al., 1980), and by malate, ethanol and N,N,N, N -tetramethyl-p.phenylenediamine ascorbate (10 mM) (Lindmark, 1980).

The rate of oxygen consumption by microbes is often referred to as respiration; but this term more correctly applies to all reactions involved in both O_2 utilisation and CO_2 production (Davis et al., 1980). In G. intestinalis; the consumption of oxygen is indicative of the unique carbohydrate metabolism of this protozoan.

As in most protozoa, glucose is the endogenous substrate for energy production via a classic glycolytic pathway. Lindmark (1980), using standard enzyme assays, has demonstrated the activity of two glycolytic enzymes (hexokinase and aldolase) in trophozoite suspensions. In addition, iodoacetamide (an acetylating agent) inhibits trophozoite oxygen consumption at a concentration of 20 mM (Lindmark, 1980); a further indication of active glycolysis.

G. intestinalis partially oxidises carbohydrates to produce organic end-products (acetate and ethanol), and CO_2, both aerobically and anaerobically (Lindmark, 1980); although the proportions in which these compounds are released varies with culture conditions. Under normal culture conditions; which are usually aerobic to a limited degree; end products of carbohydrate metabolism are formed to the following levels;

ethanol	4.8 ± 0.6 nmol/min/mg protein
acetate	23.7 ± 2.7 nmol/min/mg protein
CO_2	20.1 ± 1.8 nmol/min/mg protein

while, under anaerobic conditions the following levels of end product have been recorded:

ethanol	7.0 ± 1.3 nmol/min/mg protein
acetate	2.8 ± 0.4 nmol/min/mg protein
CO_2	6.4 ± 1.6 nmol/min/mg protein

where; under anaerobic conditions, oxidised end product is removed, as acetaldehyde is reduced to ethanol. (Lindmark, 1980).

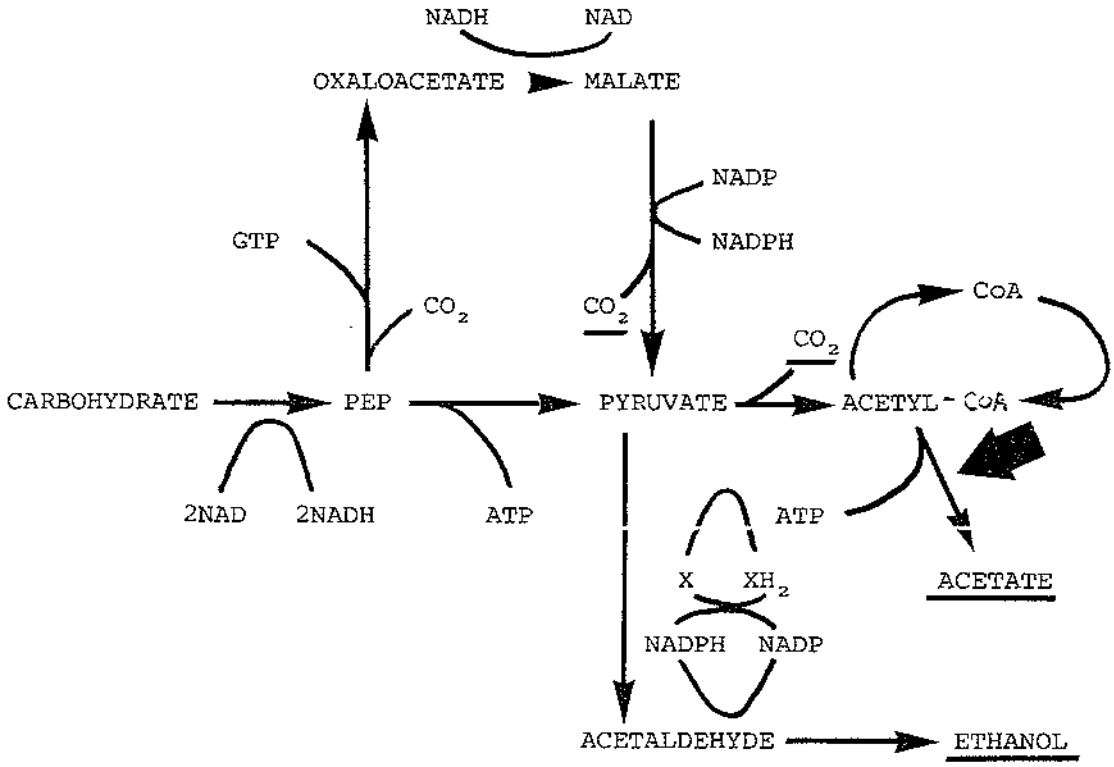
Carbohydrates are utilised by G. intestinalis in the absence of a functional Kreb's cycle and cytochrome-mediated oxidative phosphorylation (Friend, 1966; Lindmark, 1980; Weinbach et al., 1980; Lindmark and Jarrol, 1982), as indicated by the inability of Kreb's cycle intermediates to stimulate respiration; and the ineffectiveness of Kreb's cycle inhibitors (ie rotenone, cyanide and dinitrophenol) in blocking oxygen consumption by trophozoites (Lindmark, 1980).

Paramagnetic resonance spectroscopy (EPR) studies demonstrating spectra characteristic of Fe-S centres, and the detection of non-heme-iron and acid labile sulphur in disrupted trophozoite suspensions (Weinbach et al., 1980) have indicated, instead; the involvement of iron-sulphur centres in electron transport and energy production. In view of the detection of acid-labile (free) flavins in trophozoite suspensions, (Weinbach et al., 1980); and the observed inhibition of respiration by Quinacrine; a flavoprotein inhibitor (Lindmark, 1980; Weinbach et al., 1980) (See later discussion); the involvement of flavins in electron transport has also been implicated. Lindmark (1980) and Weinback et al., (1980) have demonstrated that catabolism of carbohydrates to produce energy occurs via substrate level phosphorylation and a flavin iron-sulphur protein mediated electron transport system (See 1.7). In addition, radiorespirometric studies have indicated that the major routes of glucose metabolism in G. intestinalis are via Embden-Meyerhof-Parnas and pentose-phosphate-pathways (Jarrol et al., 1981).

As already noted (see earlier discussion), the cellular physiology of G. intestinalis is similar to that of another aerotolerant anaerobe, E. histolytica. Like G. intestinalis; E. histolytica avidly consumes oxygen (Weinback and Diamond, 1974). The cellular organisation of these two unrelated protozoa is similar in that both lack mitochondria, mitochondrial enzymes, microbodies and heme proteins (Friend, 1966; Reeves, 1972; Weinbach and Diamond, 1974; Lindmark, 1980; Weinbach et al., 1980). The end products of glucose

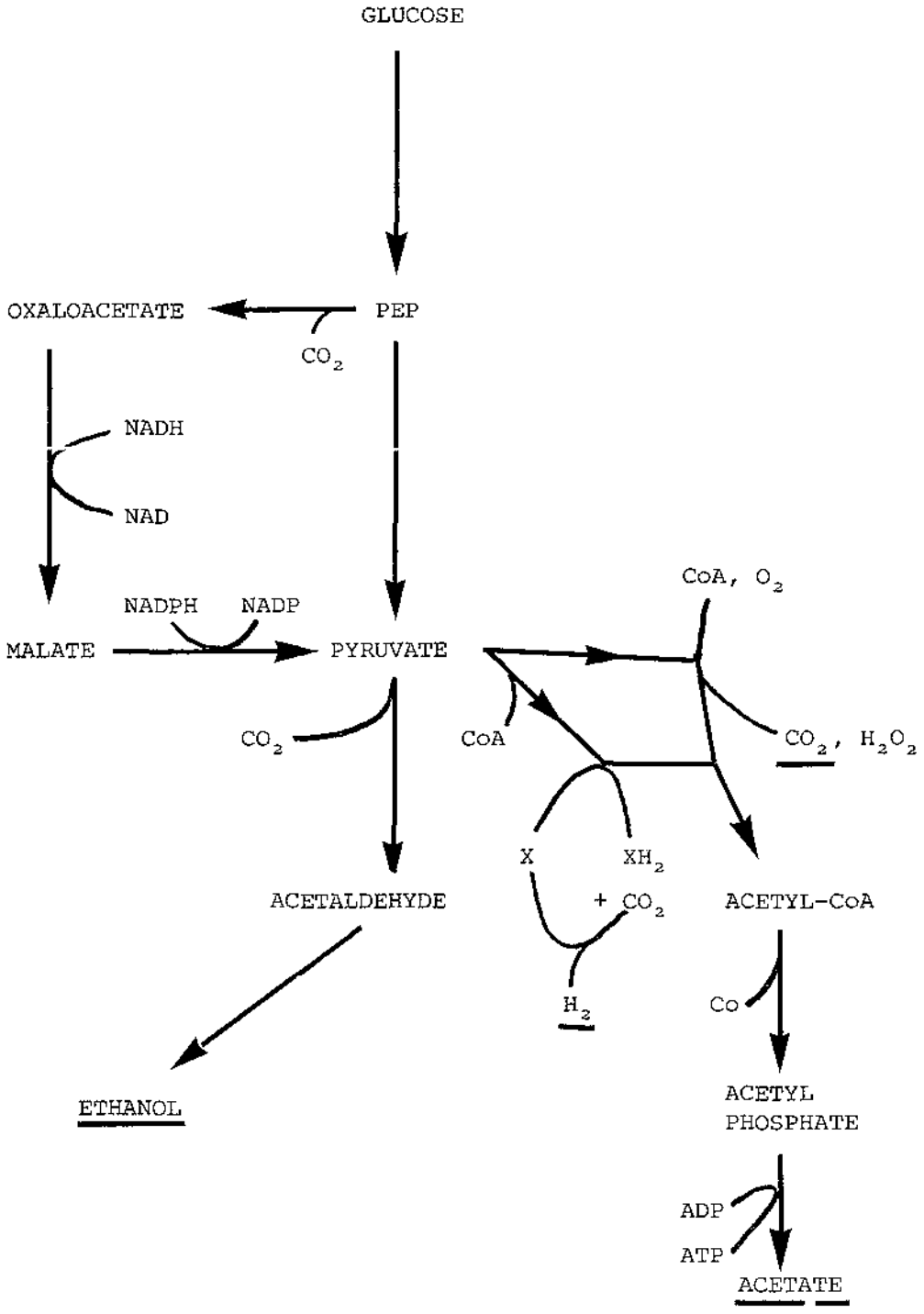
metabolism in E. histolytica are CO_2, H_2, acetate and ethanol; and; as with G. intestinalis; the rates of formation of these end products varies with culture conditions. (Reeves, 1972; Gutteridge and Coombe, 1977). (Fig 32).

Figure 31: Carbohydrate Metabolism in G. intestinalis. (From Lindmark, 1980).



NB. Acetyl CoA Synthetase marked with arrow.

Figure 32: Carbohydrate Metabolism in E. histolytica. (From Reeves, 1972; Weinbach and Diamond, 1974; Gutteridge and Coombe, 1977).



Finally; the production of acetate and ethanol from phosphoenolpyruvate is accomplished by enzymes similar to those found in G. intestinalis (See 1.7). Trichomonads also exhibit the same rather unique pattern of carbohydrate metabolism (Muller, 1976).

Generally, it is apparent that G. intestinalis and other physiologically similar protozoa lack a functional respiratory system analogous to that observed in the mitochondria of most eukaryotic cells; where there is a complete TCA cycle and the transfer of electrons via a cytochrome chain is coupled to oxidative phosphorylation. (The iron-sulphur centres demonstrated in G. intestinalis are not unique to this protozoan. Fe-S centres are crucial in most respiring cells as components of the cytochrome system. In addition, Fe-S centres are involved in the photosynthetic electron transport system of higher plants. (Lehninger, 1982).

While the current study has demonstrated the high affinity of Hast/87/MUGU/68 for oxygen at low concentrations the specific sequence of enzymes responsible for the consumption of oxygen observed; both in this study, and by previous workers, is yet to be elucidated. Gutteridge and Coombe (1977) have suggested that the activity of pyruvate oxidase (CoA acetylating) in E. histolytica may explain both oxygen consumption by this organism, and the change in end products and their proportions from aerobic to anaerobic conditions. Considering the homogeneity which exists between the carbohydrate metabolism of E. histolytica and G. intestinalis (See Figs 31 and 32); oxygen consumption in G. intestinalis may have a similar basis.

Pyruvate oxidase in E. histolytica is equivalent in activity to Acetyl CoA synthetase in G. intestinalis (Lindmark, 1980); therefore it seems reasonable to assume an analogous role for this enzyme in the mediation of oxygen consumption by G. intestinalis (See Figs 31 and 32).

As noted earlier; oxygen consumption by G. intestinalis is inhibited

by Quinacrine; the basis for this inhibition being the flavoantagonistic nature of this compound (Hellerman et al., 1946).

Weinbach et al., (1980) have noted a complete suppression of NAD(P)H oxidation in trophozoite suspensions exposed to 3.0 mM Quinacrine. In addition, a 40% decrease in oxygen uptake for the utilisation of endogenous glucose is recorded after exposure of trophozoite suspensions to 15.0 mM Quinacrine (Lindmark), 1980).

In this study, a similarly marked suppression of oxygen consumption was observed in those trophozoite suspensions which included Quinacrine (See Fig 30). The rate of oxygen consumption by trophozoites (ppm/cell/hour) was reduced up to 50% upon exposure to 1000 µg/ml Quinacrine (1.9 mM). In all cases, the concentrations of this compound required, to produce an inhibitory effect upon the respiration of G. intestinalis were in excess of the Minimum Lethal Concentration (MLC) determined for Quinacrine (See Tables IX and X, and Fig 28).

The Quinacrine MLC for G. intestinalis in vitro has previously been estimated to be 0.021-0.53 µmoles/l (Gillin and Diamond, 1981; Smith et al., 1982; Boreham et al., 1984). These Quinacrine concentrations correlate well with those recorded in this study; where trophozoites were exposed to Quinacrine in semi-solid agarose medium over 5 days incubation. Under these conditions the MLC obtained was 0.02 µg/ml - 0.04 µg/ml or 0.04-0.08 µM (See Table IX).

G. intestinalis has been described as an aerotolerant anaerobe (Lindmark, 1980; Lindmark and Jarrol, 1982). Such organisms differ from facultative anaerobes in that their metabolism remains fermentative under anaerobic and aerobic conditions ie carbohydrates are not oxidised completely to CO_2, even in the presence of air (Davis et al., 1980).

G. intestinalis is one of a small number of protozoa (see earlier discssion); which avidly consume oxygen while displaying a largely fermentative metabolism (Lindmark, 1980; Weinback et al., 1980; Jarrol et al., 1981); where carbohydrates are oxidised incompletely

to produce ethanol, acetate and CO_2 (Lindmark, 1980).

The biological significance of this oxygen consumption, and the observed variation in the formation of end products, dependant on culture conditions (see earlier discussion); is yet to be elucidated.

Active respiration by G. intestinalis may function as a 'scavenging system' to remove oxygen from the environment, as has been suggested for E. histolytica (5.4). (Weinbach and Diamond, 1974).

While it seems unlikely that oxygen is necessary for energy production, since the energy needs of G. intestinalis are met by glycolysis; (Lindmark, 1980); oxygen may be required for various biosynthetic processes; a role for oxygen similar to that proposed by Weinbach and Diamond, (1974) in E. Histolytica.

APPENDICES

Glassware:

All glassware used for routine culture maintenance and experimental work was cleaned in a chromic acid bath for 24 hours before use.

Glassware was sterilized by autoclaving at 15 lb/sq in for 15-30 minutes.

Maintenance of Axenic Cultures:

i. Cultures were incubated at 37°C in 13 x 100 mm Kimax borosilicate, screw-top culture tubes for 72-96 hours; or until trophozoites formed a monolayer on the culture tube surface.

ii. Expired TY1-S-33 was discarded and replaced by 2-3 ml of chilled, sterile HBSS.

iii. The culture tubes were then laid horizontally for 1-2 minutes to allow trophozoite detachment to take place.

iv. After gentle agitation of these tubes; 0.3-0.4 ml of the resultant trophozoite suspension (4.0×10^5 - 1.0×10^6 cells/ml); was transferred by pasteur-pipette to two 13 x 100 mm Kimax culture tubes.

v. Both these tubes were filled completely with TY1-S-33 to minimise exposure of trophozoites to oxygen.

vi. Culture tubes were then incubated horizontally at 37°C for 72-96 hours, at which stage the above procedures were repeated.

Preparation of Quinacrine dihydrochloride Solution

i. Quinacrine dihydrochloride was combined with PBS to the following concentrations (μg/ml):

10, 500, 1000.

ii. 5 ml aliquots of this solution were filter-sterilized and stored at 0°C.

Table XI: The Effect of Incubation Temperature on Growth of Bris/83/HEPU/106.

Incubation Temperature (°C)	Growth Rate (gen/hr)
25	_a
30	-0.017
37	0.083
40	0.038
45	_a

Table XII: The Effect of Incubation Temperature on Growth of Hast/87/MUGU/68.

Incubation Temperature (°C)	Growth Rate (gen/hr)
25	-0.007
30	0.020
37	0.092
40	0.045
45	_a

a - indicates rapid death of cultures.

Table XIII: The Effect of pH on Growth of Bris/83/HEPU/106.

pH	Growth Rate (gen/hr)
6.00	0.050
6.25	0.055
6.50	0.061
6.75	0.073
7.00	0.084
7.25	0.088
7.50	0.080
7.75	0.067
8.00	0.019

Table XIV: The Effect of pH on Growth of Hast/87/MUGU/68.

pH	Growth Rate (gen/hr)
6.00	0.056
6.25	0.065
6.50	0.072
6.75	0.085
7.00	0.107
7.25	0.095
7.50	0.051
7.75	0.026
8.00	0.005

Table XV: The Effect of Reducing Conditions on Growth of Bris/83/HEPU/106.

L-cysteine conc (% w/v)	O-R Potential (mV)	Growth Rate (gen hr^{-1})	% of Growth Under Standard Culture Conditions
0.00	-114.7	-0.036	-37%
0.05	-191.5	0.064	65%
0.10	-243.6	0.097	99%
0.15	-272.4	0.098	100%
0.20	-289.8	0.105	107%
0.25	-302.1	0.112	114%

Table XVI: The Effect of Reducing Conditions on Growth of Hast/87/MUGU/68.

L-cysteine conc (% w/v)	O-R Potential (mV)	Growth Rate (gen hr^{-1})	% of Growth Under Standard Culture Conditions
0.00	-114.7	-0.048	-48%
0.05	-191.5	0.08	81%
0.10	-243.6	0.093	94%
0.15	-272.4	0.099	100%
0.20	-289.8	0.103	104%
0.25	-302.1	0.102	103%

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