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ISOENZYME CHARACTERIZATION OF TRICHOMONAD PARASITES

by

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Dedicated to my wife

Sayeda,

my daughter Doaa,

my son Ahmed, and

the memory of my father.

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ABSTRACT

A review of the literature on the history of *Trichomonas vaginalis* and studies on differences among *T. vaginalis* strains was carried out. A need for the study of the characteristics that might help identification of trichomonads in general and *T. vaginalis* in particular was noted.

Studies on isoenzyme variants revealed four enzymes that could be useful in distinguishing between different trichomonads and among different strains of *T. vaginalis*. These were lactate dehydrogenase, malate dehydrogenase, hexokinase and glucose phosphate isomerase. Using these four enzymes, it was possible to divide most strains of *T. vaginalis* into 5 groups. The importance of the isoenzyme results is discussed.

It was possible to seek correlation between isoenzyme differences and other biological properties such as concanavalin A-induced agglutination and generation time of the parasites; strong correlation between these properties and the previously divided group were found.

Isoenzyme studies on flagellates isolated from faeces of monkey showed them to be very different from *T. vaginalis*; electron microscopy revealed that these organisms are probably a *Trichomitus* species.

The results of isoenzyme and other studies on some unusual T. vaginalis strains are also discussed.

Investigation into isolation, cloning and staining for light microscopy studies were also undertaken.

CONTENTS

PART I

INTRODUCTION AND LITERATURE REVIEW

A. INTRODUCTION AND LITERATURE REVIEW

A.1 HISTORY AND NOMENCLATURE OF TRICHOMONAS VAGINALIS

In 1836, Donné, a French investigator, discovered microscopic animals of a form previously unreported in the abnormal discharge of a woman suffering from vaginitis. He described their size as approximately that of pus cell and their form as tending to roundness but observed that they assumed diverse shape. He also described a flagellum-shaped anterior appendage, suggestive of a tube, which moved with great rapidity. Several cilia in motion were seen by Donné along the side of the parasite as well as on the posterior appendage. According to the morphologic concepts of that time, Donné felt that, because of the anteriorly directed structure, the organism resembled the genus Monas while the cilia suggested the species Tricodes. He therefore designated a new genus and species of Protozoa which he named Trico-monas vaginalé.

In 1837, Donné (cited from Trussell, 1947) believed that the protozoan was related to syphilis and gonorrhoea, although he had found several patients with chronic vaginal discharge in whom the parasite could be demonstrated, but who were apparently free from venereal disease. Some years later (in 1844; cited in Trussell, 1947) he changed his opinion of the relationship of this parasite to syphilis and gonorrhoea when he found the

parasite in healthy women.

Apparently, Ehrenberg (1838) was the first to emend Donné's nomenclature to Trichomonas vaginalis which has come into common usage.

Donné's discovery was questioned for many years and approximately 80 years had to pass for the complete acceptance of his findings because many authors did not believe that these "microscopic animals" were true parasites and some even believed that they were epithelial cells detached from the uterus.

Scanzoni and Kolliker (1855) found the parasite in many pregnant and nonpregnant women with purulent vaginal discharge, thus reviving Donné's findings. Even so, the parasite was generally regarded as harmless until 1916, when Höhne (1916) found a constant association of the protozoan with certain clinical findings. As a result of this work, much attention has been given to the parasite and to its medical importance. A large amount of experimental and clinical work has been conducted to elucidate its pathology, pathogenesis and association with abnormal vaginal discharge.

A.2 Taxonomy of the Order Trichomonadida

Trichomonas vaginalis is a member of the phylum Protozoa, sub-phylum Sarcomastigophora, super-class Mastigophora, class Zoomastigophorea, order Trichomonadida (Honigberg et al., 1964a). A detailed discussion of the

characteristics and evolutionary relationships of the members of the order Trichomonadida is given by Honigberg (1963) and Brugerolle (1976); an outline classification is given below.

ORDER TRICHOMONADIDA: General characters:

Generally 4 to 6 flagella, one of which is recurrent, per mastigont system; undulating membrane, if present, associated with recurrent flagellum; axostyle and parabasal body (= Golgi body) in each mastigont; division spindle extranuclear; sexuality unknown; true cysts unknown; nearly all parasitic or symbiotic.

Four families, each with several sub-families have been described (Brugerolle, 1976). Listed below are the four families, their sub-families and brief details of representative genera and species.

(A) Family MONOCERCOMONADIDAE

Sub-family MONOCERCOMONADINAE

Monocercomonas (large intestine of reptiles), *Hexamastix*, *Tricercomitus*

Sub-family PROTRICHOMONADINAE

Histomonas (*H. meleagridis*, caecum of turkeys and other galliform birds)

Parahistomonas (*P. wenrichi*)

Sub-family DIENTAMOEBINAE

Dientamoeba (*D. fragilis*, large intestine of man)

Sub-family CHILOMITINAE

Chilomitus

Sub-family HYPOTRICHOMONADINAE

Hypotrichomonas, *Pseudotrichomonas*

(B) *Family TRICHOMONADIDAE*

Sub-family TRICHOMONADINAE

Trichomonas (*T. vaginalis*, urogenital tract of man; *T. gallinae*, mouth, pharynx, oesophagus, crop of pigeons, turkeys and a wide variety of birds; *T. tenax*, oral cavity of man)

Tetratrichomonas (*Tet. gallinarum*, caecum of turkeys, chickens and many other gallinaceous birds)

Pentatrichomonas (*P. hominis*, large intestine of primates, including man, cats, dogs and rodents)

Trichomitus

Sub-family TRITRICHOMONADINAE

Tritrichomas (*Tri. foetus*, urogenital tract of cattle; *Tri. suis*, nasal cavity, stomach and intestine of swine; *Tri. muris*, large intestine of rodents)

Sub-family TRICHOMITOPSIINAE

Trichomitopsis, *Pseudotrypanosoma*

Sub-family PENTATRICHOMONOIDINAE

Pentatrichomonoides

(C) *Family DEVESCOVINIDAE*

Devescovina

(D) *Family CALONYMPHIDAE*

Calonympha

A.3 Structure of Trichomonas vaginalis

A.3.1 Light microscopy

There are several descriptive accounts of Trichomonas vaginalis based on light microscopic observations (e.g. Hawes, 1947; Honigberg and King, 1964; Powell, 1936; Wenrich, 1931, 1944b, 1947). The report published by Honigberg and King (1964) includes photomicrographs, line diagrams and descriptions of living organisms as seen with the aid of phase-contrast optics and of fixed cells stained with protargol.

A.3.1.1 Shape

The shape of Trichomonas vaginalis is variable both in living organisms and in fixed and stained preparation. It has been established that the shape and size of trichomonads are affected by the fixation method (Abraham and Honigberg, 1964; Glebski, 1969a; Honigberg and King, 1964). Some strains appear on average rounder than others, although individual variations can be seen among members of a single population. The shape of flagellates seems to depend also upon the osmotic pressure (Jirovec and Petru, 1968; Kurnatowska, 1962); in hypertonic sodium chloride solution the trichomonads tend to be spindle-shaped, while in hypotonic saline they round up and become vacuolated. Organisms in vaginal smears

are more elongated and longer than those found in cultures (Glebski, 1969a; Honigberg and King, 1964).

The typical actively swimming organisms, especially those from in vitro cultures, tend to be ellipsoidal or ovoid. Some strains tend to be quite amoeboid, and this tendency is especially pronounced in the organisms in vaginal secretions and in freshly isolated cultures (Nielsen, 1975). All strains, however, have the capacity for the formation of pseudopodia-like extensions which are employed in feeding and serve for attachment of the flagellates to various solid objects. Normal organisms always have four anterior flagella which originate in an anteriorly located kinetosomal complex, and after protargol-staining they are seen to end in small rods or knobs.

A.3.1.2 Undulating membrane

The undulating membrane and the costa originate from the kinetosomal complex somewhat posterior and dorsal to the anterior flagella. The membrane is typically shorter than the body and relatively low. The external free margin of the undulating membrane consists of the "accessory filaments" and is attached to the recurrent 5th flagellum, which does not continue beyond the end of the undulating membrane as a free posterior flagellum. The costa is accompanied on each side by a row of paracostal granules which are visible quite clearly in hematoxylin-stained preparation and on living

organisms viewed in phase-contrast system. The anterior part of the axostyle is spatulate (axostylar capitulum) and closely applied to the nucleus. The capitulum connects anteriorly to crescent-shaped pelta, seen most clearly after protargol-staining.

The trunk of the axostyle is relatively slender. It passes approximately through the centre of the cell, projects for some distance from the posterior body surface and tapers more or less gradually to a point.

A.3.1.3 Hydrogenosomes

One of the most striking features of Trichomonas vaginalis is the constant arrangement of the paraxostylar granules. Recently (Lindmark et al., 1975; Lindmark and Müller, 1973), it has been found that most of the paraxostylar and paracostal granules are anaerobic energy-generating organelles which have been named hydrogenosomes.

A.3.1.4 Parabasal apparatus

The parabasal apparatus consists of parabasal body which is seen as v-shaped in most strains and associated with one or more parabasal filaments. Electron microscope shows it to be morphologically a Golgi zone.

A.3.1.5 Nucleus

In living organisms the nucleus is situated close to the anterior end of the body and it is ellipsoidal or ovoid. However the nuclei often appear quite elongate.

A.3.1.6 Cytoplasm

In the cytoplasm there are inclusions of varying size: for example hydrogenosomes, glycogen granules and chromatin granules.

A.3.1.7 Size

The biometrics of Trichomonas vaginalis were worked out by Kurnatowska (1964, 1966) who determined the length, breadth, surface of projection volume, shape index (Length/breadth ratio) and the plasmonuclear surface and volumetric indices. It was found that the size of Trichomonas vaginalis varies from one patient to another and even among populations from the same patient at various stages of infection. Statistically significant differences exist in the biometric characteristics of flagellates from women with asymptomatic, acute and chronic infection (Jirovec and Petru, 1968). Winston (1974) has found a relationship between pathogenicity and size of Trichomonas vaginalis. Patients infected with

large trichomonads (16 µm in length) had vaginal discharges and inflammatory changes of vaginal squamous epithelium.

Using protargol-stained preparation Honigberg and King (1964) made measurements for ten strains of T. vaginalis (two from vaginal secretions and eight from axenic cultures) and they found the length and width were ranging from 4.5 to 19 µm and from 2.5 to 12.5 µm respectively.

Because of these observations, Honigberg (1978b) suggested the need for great caution in attempts to differentiate species of Trichomonadinae on the basis of size or shape, and even more in attempts at differentiating strains within a single species. Such differentiation should be based on studies of flagellates grown under comparable conditions, in as far as possible identical culture medium and retrieved from populations in closely comparable growth phases.

A.3.2 Electron microscopy

Since 1959, much work on the ultrastructure of T. vaginalis has been published, for example: Inoki et al. (1960), Ludvik et al. (1961), Nielsen et al. (1966, 1975, 1976, 1978), Nielsen (1975, 1976a, 1976b), Smith and Stewart (1966),

Ovcinnikov *et al.* (1974, 1975), Brugerolle *et al.* (1974). An ultrastructural characteristics of trichomonad flagellates is the absence of morphologically recognizable mitochondria; Smith and Stewart (1966) confirmed Inoki's (1960) original observation that these organelles are absent. One of the most striking features of *T. vaginalis* is the presence of microbody-like parabasal and paraxostylar granules. Linmark and Müller (1973) described similar microbody-like organelles in *Trichomonas foetus* as a membrane-bound organelle characterized by rather uniformly granular matrix with occasional denser inclusion and membranous internal structures. Recently Lindmark *et al.* (1975) demonstrated in *T. vaginalis* that these granules are the site of anaerobic enzymes participating in metabolising pyruvate with formation of H_2 and CO_2. They are now generally known as hydrogenosomes.

With the aid of transmission electron microscopy Nielsen *et al.* (1966) published an extensive description accompanied by detailed interpretation of the structure of a trichomonad. The work published by Ovcinnokov *et al.* (1974, 1975) on electron microscopy studies of *T. vaginalis* has been heavily criticised by Honigberg (1978b) because of the authors' interpretation of their findings.

Different circumstances can affect the ultrastructure of *T. vaginalis*. For instance,

Nielsen (1975) studied the ultrastructure of T. vaginalis before and after transfer from vaginal secretion to Diamond's TYM (1957) medium. He found that during the first 3-4 weeks in vitro, the fine structure of all cells was changed. The shape of the nucleus, the size of the Golgi apparatus and adjacent vesicles, the fine structure of chromatic granules and the mastigont were all changed when compared to the organelles of the originally isolated cells. He found also in the cells in vitro that the "ectoplasm" (see below) had disappeared, the glycogen granules had become sparse and the number of free cytoplasmic ribosomes had increased. The "ectoplasm" layer is the peripheral part of the ctyoplasm; it forms the pseudopodia that remain in contact with the desquamated cells in vaginal discharge. This layer was also described by Nielsen and Nielsen (1975) from human biopsy specimens in which the trichomonads were found applied to the vaginal epithelium; and by Brugerolle et al. (1974) in organisms recovered from visceral lesions caused in mice by intraperitoneally inoculated trichomonads. However, the study of Nielsen and Nielsen (1975) gives no direct information on morphological differences between virulent and avirulent T. vaginalis cells.

Recently Nielsen (1976b) compared the fine structure of cells of T. vaginalis obtained from the exponential phase of growth and from stationary

cultures. He found that the number of free cytoplasmic ribosomes was higher than that in cells from stationary cultures. The volume of the Golgi region and of food vacuoles were approximately twice as large in logarithmic phase. The round and slightly ovoid chromatic granules were smaller and the elongated granules were more numerous in logarithmic growth phase. In flagellates in both phases the cell membrane was covered with patches of filamentous material which contained polysaccharides and glycoproteins, seen also in trichomonads from vaginal discharges (Nielsen, 1975) and in those from visceral lesions of intraperitoneally inoculated mice (Brugerolle et al., 1974).

Ings et al. (1974) found that the nucleic acid synthesis of T. vaginalis cells was strongly inhibited by metronidazole in low concentration. Recently Nielsen (1976a) studied the in vitro effect of metronidazole on the ultrastructure of T. vaginalis and found the fine structure of the cytoplasm was changed 30 to 60 minutes after the addition of metronidazole. Also, proportionally, the number of polyribosomes had decreased whereas the number of single ribosomes in the cytoplasm had increased and the electron density of the cytoplasmic matrix was higher.

Fine structure studies of T. vaginalis have also been published demonstrating phagocytosis

(Nielsen, 1970) and host-cell damage (Nielsen and Nielsen, 1978).

A.4 Reproduction of Trichomonas vaginalis

A brief account of this process was given by Reulling (1921) who saw division and migration of the blepharoplast followed by constriction of cytoplasm to form two daughter cells. Later Lynch (1922) described division as mitotic in character. The most extensive light microscopic study of the division process in T. vaginalis was published by Powell (1936). According to this report four chromosomes appear during the mitotic division and paradesmosome connects the daughter blepharoplasts which function as centroblepharoplasts during division. Two flagella are said to go to each daughter cell and two new flagella grow out of each blepharoplast to restore the full number of four.

Recently, Brugerolle (1975) reported the result of an electron-microscopic investigation of division and morphogenesis in the urogenital trichomonad of man. Briefly, he considers the division process to represent "cryptopleuromitosis" (a process of division which has been illustrated in detail by Hollande and Caruette-Valentin, 1971, 1972). The first manifestation of this division is the appearance

of a new kinetosome followed by appearance of two "atractophores" (Camp et al., 1973) which are connected to the kinetosomes and to parabasal filaments. Elongation of a bundle of microtubules, extending between the division poles is responsible for the separation of the daughter mastigonts. Further, chromosomal microtubules extend from each of the atractophores towards the nucleus and attach to the centrosomes which are situated on the nuclear envelope. A new axostyle is formed near the kinetosomes.

Multiple fission forms have been observed by many investigators. Marchand in 1894 was the first to report them and described a large trichomonad with three tufts of flagella apparatus and four nuclei. Trussel (1947) reported that such forms develop occasionally in his routine stock cultures of bacteria-free T. vaginalis. Also, Hoffmann and Malyszko (1966) described multiple division and polynuclear T. vaginalis in one out of 22 women examined before treatment and in 6 women on the third day of treatment with metronidazole. Bishop (1931) reviewed in considerable detail the occurrence of multiple forms which he called "somatellae". However, it is not definitely known that these multiple fission forms do divide into normal organisms. They might well be aberrations and the unfavourable environmental conditions that suppress cytokinesis (Honigberg, 1963)

may explain increase in number of polynuclear forms. The generation time of logarithmic phase culture for 9 strains of T. vaginalis was found by Kulda et al. (1970) to range from 6 hours to 13 hours and 30 minutes. Nielsen (1976b) found the generation time was 3½ hours for his strain No. 1711. Farris and Honigberg (1970) and Honigberg (1961) reported generation time between 5 and 10 hours but in another report, Honigberg (1978b) reported generation time up to 16 hours.

So far, there is no evidence of true cystic stage during the life cycle of T. vaginalis and cyst formation mentioned in the various publications reviewed later, has been the subject of controversy. There is no confirmation of sexual reproduction of trichomonads including T. vaginalis. Further studies would however be needed before the process of division in trichomonads is fully understood.

A.5 Motility of Trichomonas vaginalis

T. vaginalis moves rapidly through the pus cells and debris by the lashing action of its anterior flagella and the sweeping movement of its undulating membrane. All the strains have the capacity to form pseudopodia-like extensions, which are used in feeding or in attachment but not for amoeboid movement.

A.6 The cyst problem

The existence of a cyst stage during the life cycle of T. vaginalis has been a subject of controversy. Jirovec and Petru (1968) reported that most of the controversy which existed was based on the demonstration of structures that resembled cysts, the resistance of the organism to chilling, failure of some cases to respond to treatment, and the unexplained mode of transmission of the parasite. Some spherical, motionless trichomonads can be found in vaginal secretions and especially in the urethral secretion of male as well as in old cultures and under adverse conditions. These round forms never have any flagella or a distinguishable cyst membrane, well known characteristics of Amoeba or Giardia cysts.

Jirovec and Petru (1968) and Honigberg (1963) suggested that these formations seem to be degenerate trichomonads destined to die unless they are rapidly transferred into a culture medium or even into the genital ducts of the opposite sex.

Recently, round forms of T. vaginalis were described in detail by Trevaux et al. (1978). Catterall (1977) summarised the discussion at the International Symposium on Genitourinary Trichomoniasis, Paris (1977). He reported that the round forms became the main subject of discussion at the symposium. He continued, "... some speakers thought they could be

artefacts and were not to be related to Trichomonas vaginalis, others thought that they were dying cells and therefore of little significance, but some felt that they represented a stage in the cycle of developments of the organisms and were of pathological significance. There was considerable discussion on whether trichomoniasis should be diagnosed from the finding of round forms alone as no observer had seen these develop into classical forms of Trichomonas vaginalis. However, the opinion so far prevails that cysts are not formed by Trichomonas vaginalis and the parasite exists only in the trophozoite form."

A.7 Infection sites

A.7.1 In women

The most frequent and typical site of T. vaginalis infection is the vagina. Grys (1966) investigated 134 cases of infected women to assess the distribution of T. vaginalis in the female genital organs. He showed that the parasite does not spread beyond the external opening of the cervix. He assumed that the cervical mucus serves as a barrier to the entrance of trichomonads.

Infection of the lower urinary tract has been recorded by several investigators. Chappaz and

Bertrant (1965) found T. vaginalis in this site, but thought that infection of the ureters and renal pelvis was quite rare. Other investigators, for instance Trussell (1947) considered the lower parts of the urinary tract to constitute a frequent site of infection.

In studying localization in the genitalia, Grys (1973a) examined 1249 infected women; he found 2.81% in whom spread had occurred beyond the vagina to involve Skene glands, the urethra and in some instances Bartholin's glands. In 1966 the same author had found T. vaginalis in the bladder accompanied by an increased number of inflammatory cells in the sediment of the urine, which he suggested could be employed as a diagnostic sign in case of trichomoniasis. He also suggested that catheterization can introduce the organism into the bladder from infected urethra. Later however he reported trichomonads in the urinary bladder of only 14.13% of women with vaginal infection (Grys, 1973a).

Soszka et al. (1973c) examined 42 cases of infected women and found in 5 cases T. vaginalis present in fluid from the urethra and in 2 cases a single mobile organism in urine sediments. They suggested that the infection of the urethra is secondary process and that the urinary tract does not provide an environment advantageous for the development of the parasite.

Experimentally, infections of the urinary tracts by T. vaginalis were made in a group of 48 female and 22 male guinea pigs by Soszka et al. (1973b). They found that the bladder, renal pelvis, peritoneum and vagina were infected. However, the dissemination of T. vaginalis although common in laboratory mammals inoculated with pure cultures, seems to be very rare in women. Hoffmann et al. (1966) reported a very interesting case, in which T. vaginalis was cultured from the vagina of a patient suffering from generalized cancer. After the patient's death, the same organism was cultivated from the cancerous foci in the lungs and liver; from the spleen and from the vaginal mucus membrane. Serological analysis before and after death indicated that the Trichomonas spp. cultivated after death was identical with that cultivated during the patient's life. It is not known what route had been followed by the parasite in invading the internal organs. In an attempt to find T. vaginalis in the internal genital organs, Swierz et al. (1964) examined the oviducts (removed for other reasons) from women who exhibited longstanding trichomoniasis. They were able to isolate T. vaginalis from the oviduct of two out of four cases among four women. Grys (1973a) did not find T. vaginalis in the oviducts or in ovaries and he stated that these sites are not ecological foci of T. vaginalis. The organisms do not as

a rule ascend into the uterine cavity or fallopian tubes (Catterall, 1972), and parasitaemia does not occur (Ackers et al., 1975).

A.7.2 In men

The urethra and prostate glands are the most frequent sites of infection; involvement of testes has also been reported and infection of the epididymis cannot be considered rare (Jirovec and Petru, 1968; Siboulet, 1966; Sluki, 1966). In uncircumcised men, trichomonads were found in the subprepucial sac.

A.8 Signs and Symptoms

Between the discovery of T. vaginalis by Donné (1836) and Höhne's (1916) report, this organism was regarded by medical men and parasitologists as a harmless commensal. However, there is much evidence that T. vaginalis may cause symptomatic disease in women and, less frequently, also in men.

A.8.1 In women

Many infected women are asymptomatic. Trussell (1937), as well as many other workers, thought that asymptomatic cases exceeded in number the frankly symptomatic ones. In some infected women there is a

copious, frothy, greenish yellow or creamy white discharge from the vagina and introitus. The discharge may be malodorous. Due to the irritating effect of the abnormal discharge, there is soreness and pruritis of the vulva, which become red and oedematous followed by excoriation of the skin of the inside of the thigh. This reaction may lead to secondary infections. Reddening of the vagina and the vaginal portion of the cervix is frequent and the inguinal lymph nodes tend to enlarge and are tender to touch.

Abnormal vaginal bleeding, including blood-stained discharges and spotting as well as post-coital bleeding are said to be far more frequent in women harbouring T. vaginalis. Terzano et al. (1969) suggest that whenever bleeding not explainable by the presence of conditions known to cause these symptoms (e.g. carcinoma, myoma and polyps) occurs, T. vaginalis should be looked for.

In acute cases, haemorrhagic spots may be found on the walls of the vagina and the cervical mucosa. Because of the typical appearance of the inflammatory process in the vagina and cervix, Holtroff (1966) suggested that colposcopy can be employed to diagnose trichomoniasis; the colposcopic picture in active trichomoniasis has been described (Kolstad, 1965; Koss and Wolinska, 1959; Kozicka, 1966).

Chronic discharges may encourage the formation

of genital warts (condyloma accuminata caused by Papovavirus) which may persist for many months and then disappear as rapidly as they arose (Walter and Israel, 1974); also chronic congestion may result in menorrhagia and menstrual pain (Honigberg, 1978b). The signs and symptoms are exacerbated during menstruation, pregnancy and puerperium (Schofield, 1972; De Leon, 1971; Nielsen and Nielsen, 1975; Honigberg, 1978b).

A.8.2 In men

Men, more frequently than women, are asymptomatic carriers of T. vaginalis. Jira (1958) classified urogenital trichomoniasis in men into three types:

(a) primary acute: characterized by rapid onset and accompanied by a copious urethral discharge;

(b) primary subchronic: with slow onset and characterised by relatively slight discharge;

(c) primary latent: very frequent and without any symptoms.

It is possible that types (a) and (b) may progress to type (c). Riba (1931) reported two cases of trichomonae urethritis in men in whom the external meatus was covered by a mucoid frothy discharge containing

Trichomonas. The prostate was somewhat enlarged and tender, and Trichomonas was found in the prostatic secretion. Generally in chronic cases there may be a slight itching sensation inside the penis or slight moistness at its tip. In acute trichomonad urethritis and prostatis, the picture may resemble gonorrhoea and non-specific urethritis.

A.9 Complications

A.9.1 In women

Infection of the Skene ducts, bartholinitis, urethritis and cystitis are among the complications of trichomoniasis in women. The possible effect of urogenital trichomoniasis on fertility in women was examined by Grys (1973b). He pointed out that, since typically the parasite does not inhabit the upper parts of the urogenital tract, it cannot have directly harmful effects upon the ova or upon fertilization. On the other hand it was thought that toxic products of the parasites could damage the entire urogenital system. He also suggested that inflammation of the vagina can exert injurious effects on the sperm. It seems that typically trichomoniasis does not prevent pregnancy or cause abortions in women (Trussell, 1947) in the way Tritrichomonas foetus does in cows. There does not seem to be an increased frequency of T. vaginalis infections in infertile women (Grys, 1973b).

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A.9.2 In men

Complications of trichomoniasis in men may include prostatitis, balanoposthitis, urethral stricture, cystitis, letreitis, cowperitis, epididymitis and even involvement of testes. Rebhun (1964) reported an unusual and fascinating case of recurrent chronic bronchitis in a man that was traced to his practice of oro-genital contact with infected women before copulation. In this case, it was proved by repeated wet film examination of sputum, that the patient was infected with and allergic to T. vaginalis. However, in some instances, male sterility has been attributed to trichomoniasis; and chronic discharges can encourage the formation of urogenital warts (Honigberg, 1978b).

A.10 Non-sexually-transmitted Female Trichomoniasis

Older and recent investigators have emphasized the fact that T. vaginalis is very rare in girls and virgins but that the incidence of infection rapidly increases after defloration. However trichomonads may occasionally be acquired during birth if the mother is infected with T. vaginalis. Another possible source of transmission of T. vaginalis to newborn babies is from fomites under highly unhygienic conditions.

Komorowska et al. (1962, 1964) found infections with T. vaginalis in newborn babies and in girls over the age of 10 years were more frequent than in girls under 10. They reported that of 35 newborn babies up to 3 weeks old, 17.2% were positive for T. vaginalis; of 1101 girls up to the age of 10 years, 0.8% were positive; and of 870 girls past the age of 10, 10.4% were positive. In another report, Sagone (1973) observed Trichomonas infection in virgins in 2.09% of the cases examined. Crowther (1962) described two well documented cases of T. vaginalis in infancy at 31 days and 6 weeks old. Both of them were found to have vaginitis with discharge. T. vaginalis was found in the vaginal swab and urine taken at the same time. However, girls sharing a bed or using the same bath and lavatory as adult infected persons very seldom become infected. Urinary infection by T. vaginalis appears to be also very rare in infancy and childhood (Jirovec and Petru, 1968).

A.11 Immunology and Serology of T. vaginalis

The immunology of T. vaginalis has occupied the attention of many investigators. Honigberg (1970) pointed out that the recent results of this work clearly reflect the presence of systemic immune response in human infection with the urogenital flagellates.

The recent finding of intracellular trichomonad in patients (Frost et al., 1961) and cell cultures (Sharma and Hongiberg, 1966, 1967) provide further evidence for the occasional intimate association between the urogenital parasites and the host organism which could lead to the formation of specific antibodies. Also, the recent findings of Ackers et al. (1975) threw the light on the presence of antitrichomonal antibody in the vaginal secretions of women infected with T. vaginalis. More specifically, Chipperfield and Evans (1972) showed a marked rise in the number of immunoglobulins-bearing plasma cells in cervical biopsies from women who had been exposed to, or infected with, N. gonorrhoea, T. vaginalis or C. albicans.

A.11.1 Antigenic differences

It became apparent during the immunologic studies of T. vaginalis that antigenic differences among the strains of this parasite existed (e.g. Schoenherr, 1956; Magara, 1957; Amino, 1958; Lancely, 1958; Teras, 1965, 1966; Kott and Adler, 1961; Hoffmann and Gorczynski, 1964; Teras et al., 1966a, b and c; Laan, 1966). The presence of different antigenic types was demonstrated by precipitation, agglutination, complement-fixation, and haemagglutination tests. The occurrence of several antigenic

types of T. vaginalis within quite limited geographical areas was suggested by Kott and Adler (1961) and Hoffmann and Gorczynski (1964). Schoenherr (1956) found serologic differences among the strains of T. vaginalis by precipitation and agglutination tests and he found the latter to be the best throughout his study. Depending on the interpretation of the end point of agglutination tests, Schoenherr (1956) suggested the presence of 3 to 5 antigenic types. With the aid of agglutination, precipitation and complement-fixation methods, Magara (1957) was able to show two antigenic types among seven strains of T. vaginalis; however Amino (1958) was unable to show (by agglutination) immunologically distinct types among various strains of T. vaginalis, all of which were considered as pathogenic on the bases of intraperitoneal mouse assay. On the other hand, his complement-fixation results revealed the presence of immunologic strain specificity with titres ranging from 1:400 to 1:3200. Lancely (1958) employed a microagglutination method and indicated the presence of at least three discrete antigenic types of T. vaginalis. In fact, Hoffmann (1966a) concluded from Lancely's results (1958) that the possible existence of four antigenic types could not be excluded. Agglutination test employed by Kott and Adler (1961) clearly showed that T. vaginalis is not homogeneous antigenically, but consists of distinct

serotypes which can be readily distinguished by direct agglutination and absorption tests. Among the 19 strains tested, there were eight distinct serotypes. They also reported that two strains of T. vaginalis being grown in axenic culture were antigenically different before bacterial contamination, but were indistinguishable after reaxenization but different from the parent cultures. They suggested that either the strains which were originally distinct had changed in the same direction as a result of contamination, or both cultures originally consisted of two strains, one of which had disappeared as a result of bacterial contamination. A third possibility is that a mutant appeared during the period of contamination and replaced the original strains. Apart from these two cases, all the other noncontaminated strains were constant in their serological reactions which were tested repeatedly during an observation period of two and a half years. They also observed that the growth of T. vaginalis in the presence of immune serum during 68 passages had apparently not influenced their antigenic constitutions. Similar apparent immutability of the serotypes of T. vaginalis upon prolonged maintenance in culture was reported by Teras (1965, 1966), Laan (1966) and Teras and Tompel (1963, cited from Honigberg, 1978b). However, the finding of Kott and Adler (1961) indicated that no single serological

method they employed (direct agglutination, haemagglutination, and cross absorption) is alone sufficient to disclose all serotypes.

The Estonian investigators (Teras, 1963a, 1965, 1966; Teras and Tompel, 1963, cited from Honigberg, 1978b); Teras et al., 1966a and b) employed cross-agglutination techniques (using both absorbed and non-absorbed immune sera) as well as complement-fixation methods to differentiate between the antigenic types of several hundred strains of T. vaginalis collected from large areas of central and eastern Europe. They found four antigenic types which they named TLR, TN, TRT and TR* with common unique antigens.

More recently, Rõigas (1975) was able to differentiate the four serotypes by the direct fluorescent antibody method. The four antigenic types demonstrated by the Estonian groups seem to be distributed very widely. For instance, Teras (1966) proved that the antigenic structure of the VP type from Czechoslovakia was identical with TLR type in Estonia and the antigenic structure of LZ strains from Hungary was identical with that TR in Estonia. However, the author also suggested that additional types would be found on further studies.

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* T = referring to Teras or Tompel
  L = referring to Laan
  R = referring to Rõigas
  N = referring to Nigesen.

Using microagglutination and haemagglutination methods as well as complement-fixation, Hoffmann and Gorczynski (1964) were able to differentiate at least three definite antigenic types among 23 strains of T. vaginalis isolated from as many persons inhabiting one district in Poland. Honigberg (1970) reported results of Hoffmann and Gorczynski (1964) showing that additional types could be recognized among the 23 strains through the employment of more sensitive techniques such as the quantitative fluorescent-antibody method (Honigberg and Goldman, 1968).

Recently, by means of disc electrophoresis in polyacrylamide gel, Andreeva and Mihov (1976) examined extracts containing water soluble proteins of ten local strains of T. vaginalis. The analysis of the electrophoregrams revealed both resemblances and differences in protein fractions, in the number, electrophoretic mobility and intensity of staining of the bands. On the basis of their results, the authors were able to distribute the strains examined by twos into five groups. They suggested that the heterogeneity in the protein composition of the groups supports the existence of antigenic differences among T. vaginalis strains.

A.11.2 Natural antibodies

Much work has been published concerning

agglutinating and lytic activities of a variety of normal sera against T. vaginalis. McEntegart (1952), Kott and Adler (1961), as well as many other investigators carefully established the baseline level of natural antibodies for T. vaginalis in normal sera before evaluating the results of their serological tests. The agglutination and lysin titres of different normal sera were found to vary in reports by different investigators and even within the results of the same investigators. For instance, Tatsuki (1957) considered human serum titres of up to 1:30 as non-specific for both agglutination and lysis against T. vaginalis. Teras (1961b, cited from Honigberg, 1978b) observed that in most instances the agglutinin titres of normal human sera were between 1:40 and 1:80. In this work he used sera derived from 40 Trichomonas-free and sexually inactive boys 15 to 17 years old. In other reports the Estonian workers (Teras et al., 1966a and b) were inclined to accept as specific only agglutination reactions with human sera diluted over 1:160. Other investigators such as Hoffmann and Gorczynski (1964) established agglutination, haemagglutination and complement-fixation titres of 1:20, 1:5, 1:5 respectively for normal rabbit sera against T. vaginalis, while Lancely (1958) observed that rabbit antisera to several strains of T. vaginalis is specific when titres exceed 1:40.

According to Samuels and Chun-Hoon (1964), one strain of T. vaginalis was agglutinated by normal calf serum at dilutions ranging from 1:8 to 1:512. Inasmuch as the agglutinating activity of this serum for T. vaginalis was not reduced significantly by absorption with Tritrichomonas augusta, and because all its activity was eliminated by absorption with the homologous antigen, the reaction was regarded as being due to specific antibodies rather than non-specific host defence factors (Samuels and Chun-Hoon, 1964). Reisenhofer (1963) studied extensively the lytic and agglutinating activities of normal male and female human sera as well as normal calf, sheep, horse, swine and dog sera. The results of this work brought out many interesting differences among these sera with regard to one strain of T. vaginalis. However, it was found that fresh human sera had strong lytic properties resulting in severe damage to living flagellates at dilutions which ranged from undiluted or 1:2 to 1:32, and that dilutions higher than 1:64 were no longer effective against the parasite. Reisenhofer (1963) reported that the sex and age of the donors had no effect on the lytic activity and no differences were observed in this regard between sera from persons who harboured the flagellate and from trichomonad-free individuals. In more detail, Reisenhofer (1963) found a higher agglutinating capacity for T. vaginalis in inactivated than in unheated normal human sera. It

is clear from this report that two distinct anti-trichomonal activities, a lytic one and an agglutinating one, are exhibited by various normal sera and that lytic activity seems to be dependent upon the presence of complement. It is not clear whether subsequent infection with T. vaginalis leads to a boosting of the existing antibody level or the eliciting of a new primary response (Ackers and Lumsden, 1978), although there is evidence that in the case of Tritrichomonas foetus infection of cows the latter is the case (Kerr and Robertson, 1953).

However, a similar situation was reported with regard to natural antibodies against Tritrichomonas augusta (Samuels and Chun-Hoon, 1964) and against Monocercomonas colubrorum, Trichomonas gallinae and Tritrichomonas foetus (Robertson, 1941). Apparently the natural antibodies present in normal sera were not type-specific. In any case, it is clear that in applying any serodiagnostic test, the baseline level of natural antibody must be carefully examined.

A.12 Serodiagnosis

Immunodiagnosis of T. vaginalis has occupied the attention of many investigators, and several methods have been employed with varying degrees of success. It seems that the contradictory results

reported by different authors may be due to the antigenic structure of the individual of T. vaginalis strains. The methods employed included: complement-fixation, agglutination (including microagglutination), haemagglutination, and fluorescent-antibody techniques. Some success was also achieved by the use of delayed-type hypersensitivity reactions. Recently, Ackers et al. (1975), using radioimmunoassay, were able to establish the presence of antitrichomonal antibody in the vaginal secretions of women infected with T. vaginalis; on the other hand, using the same technique, no detectable local antibody could be detected in genitourinary tract secretions of male contacts of infected women (Ackers et al., 1978). These results will be discussed later.

A.12.1 Complement-fixation

Wendlberger (1936), using alcohol-extracted T. vaginalis antigen derived from agnotobiotic cultures, was the first to obtain positive complement-fixation reactions, finding this in 22 out of 48 women. During this work, patients positive on serologic testing were found to harbour the urogenital trichomonad and in all of them infection was accompanied by abnormal vaginal discharges, with or without other clinical manifestations. Among the women whose sera failed to inhibit haemolysis, 10 harboured T. vaginalis, but only

two of these had any abnormal discharges. Wendlberger (1936) pointed out that, since the latter two patients suffered from active gonorrhoea, the discharges might not have been caused by the presence of the trichomonads. The remaining 16 women, negative on serologic examination, were found not to harbour the urogenital flagellates. On the basis of his finding, Wendlberger suggested that asymptomatic infections failed to evoke a systemic immune response.

Using a suspension of bacteria-free T. vaginalis in saline as antigen, Trussell et al. (1942) obtained a positive complement-fixation reaction by Kolmer's method with sera from 47.3% of 110 women who harboured T. vaginalis and presented with or without clinical symptoms. They also found 16.5% of 290 parasite-free patients were positive on serological testing. The authors did not suggest any reason why complement-fixing antibody was found in parasite-free patients. The detection of complement-fixing antibody in patients harbouring T. vaginalis but without symptoms appears to contradict the results reported by Wendlberger (1936) (see above). Stepkowski and Bartoszewski (1959), using a formalinized antigen, were able to detect a positive complement-fixation reaction in only 30% of patients who were found to harbour T. vaginalis on microscopic examination. Korte (1957) found high complement-fixation titres in

sera from patients with trichomoniasis and low titres in sera from patients with symptoms and pathologic manifestations characteristic of "persistent, chronic, or recurrent" infection. He also mentioned that the cases with high titres were readily amenable to treatment, whereas those with low titres were refractory. In the light of such correlation, Korte (1957) considered serodiagnosis is an important tool in prognosis.

Teras (1962b, cited from Honigberg, 1970), using the same test, examined 114 sera of persons found to harbour T. vaginalis on direct examination and by culture. He found that 86% gave positive reactions. He also suggested that additional positive results could be achieved through the use of different strains of T. vaginalis as antigens. Hoffmann et al. (1963) employed the quantitative Kolmer method in conducting extensive study of complement-fixation reaction in human urogenital trichomoniasis. The antigens they used were phenol-treated suspension and homogenates prepared by freeze-thawing from a mixture of 20 strains of T. vaginalis, 10 of which were isolated from women and 10 from men. No differences were observed between the results obtained with the two types of antigen. The sensitivity of the complement-fixation reaction was tested first on sera from rabbits immunized with the mixture of trichomonad strains. Among 715 persons, 275 (about

38%) were found to harbour T. vaginalis; 5 girls less than 15 years old, 115 mature women, and 155 mature men. Positive complement-fixation results were found in 2 girls (40%), 93 (81%) mature women and 62 (40%) mature men. In comparison with the clinical symptoms, Hoffmann et al. (1963) found that the complement-fixation reaction was positive most often in persons suffering from chronic trichomoniasis and least often in those showing symptoms associated with the acute form of infection. Also, the positive reaction is observed more often and has a higher titre in women than in men infected with T. vaginalis. No boys below the age of 15 were found infected with the urogenital trichomonad, and none gave a positive complement-fixation reaction. On the other hand, of the remaining 440 persons who showed no infection with T. vaginalis on direct examination and by the culture method, 36 (8.1%) gave a positive complement-fixation reaction. Among these 36 cases, 7%, were found to be girls whose mothers were infected with T. vaginalis, 4% were mature women who were treated for trichomoniasis within the preceding few years, and 16% were men who were found to have had sexual relations with women infected with T. vaginalis.

From these results, Hoffmann et al. (1963) thought it likely that some of the serologically positive but apparently parasite-free persons might have

actually harboured T. vaginalis which had been missed on examination, particularly in view of the well-known cryptic infection in male patients, in whom the urogenital flagellates often are very difficult to find on direct examination or even by culture methods. Alternatively, the results might be due to the presence of antibodies still present from previous infections.

Hoffmann et al. (1963) concluded that despite the difficulties encountered in serologic studies of urogenital trichomoniasis, the various immunological methods, including complement-fixation, deserve further attention because they hold promise for the clinician and the epidemiologist.

In the light of the results of Hoffmann's group, Honigberg (1970) reported that it would therefore appear that a positive serologic test in supposedly non-infected persons must not be considered as nonspecific. Also, the great differences between the infected and noninfected groups in the percentage of cases found positive on serologic examination and in the average titres are evidence for the specificity of the complement-fixation reaction in urogenital trichomoniasis.

In another extensive study, Jaakmees (1965, cited by Honigberg, 1970) investigated the dependence of the complement-fixation reaction on the antigenic

type of T. vaginalis (summarized in Table 1) as well as its relation to the clinical manifestations of trichomoniasis (summarized in Table 2). Trichomonads belonging to the four major antigenic types (TN, TLR, TRT and TR) recognised by the Estonian workers were employed throughout this study. In contradistinction to the results reported by Hoffmann et al. (1963), the data in Table 2 suggests a lack of correlation between the results obtained by complement-fixation reaction and the clinical form of trichomoniasis. From the results of Jaakmees (1965, cited from Honigberg, 1970), which are summarized in Table 1 and Teras et al. (1966b), some positive serologic reactions would be missed if only one antigenic type of trichomonad were employed in serodiagnostic tests. Regardless of the fact that the results of complement-fixation reaction depend on the antigen employed (homologous or heterologous serotypes), the reaction does not reveal the serotype the patient has been infected. This is because complement-fixation reaction with heterologous serotypes can frequently be as strong as with the homologous serotype.

Recently Jaakmees et al. (1966) investigated the dynamics of complement-fixation in the sera of patients treated with metronidazole. It was found that in all cases T. vaginalis disappeared from the urogenital tract not later than the fourth day of

Table 1: Dependence of the Complement-fixation Reaction on the Antigenic Type of *T. vaginalis* employed in tests*

Sera from persons infected with type	No. of sera	Positive reactions with antigens (++++ or +++)					
		TR	TRT	TN	TLR	Homo-logous	All
TR	43	43	32	33	33	43	24
TRT	29	24	29	23	24	29	20
TN	14	12	12	13	13	13	11
TLR	13	9	12	11	13	13	9
Totals	99	88	85	80	83	98	64

* After Jaakmees, 1965 (quoted by Honigberg, 1970).

Table 2: Dependence of the complement-fixation reaction on the antigenic type of T. vaginalis employed in the serodiagnostic tests and its relation to the clinical manifestation of Trichomoniasis*

Sex	Clinical manifestations	No. of sera	Positive Reactions (++++ or +++) with antigens TN	TLR	TRT	TR
Female	Acute	44	37	37	40	36
	Subacute	46	41	41	44	41
	Chronic	70	61	51	55	57
	None**	11	10	11	11	9
Totals		171	149	140	150	143
Male	Acute and subacute	12	10	9	9	11
	Chronic	39	32	27	27	30
	None**	32	27	24	27	27
Totals		83	69	60	63	68

* After Jaakmees, 1965 (quoted by Honigberg, 1970).

** "Latent" trichomoniasis; this term is commonly used by the Estonian investigators for asymptomatic cases i.e. cases without clinical manifestation.

treatment, followed by rapid regress of clinical symptoms. The complement-fixing antibodies in the sera of treated patients as well as the results of treatment were investigated in all cases, monthly during the first half and every three months during the second half of the year following the metronidazole treatment. The results obtained showed that the amount of complement-fixing antibody in the sera starts to decrease within the third month after treatment. In most cases complement-fixing antibodies completely disappeared after one year but eight cases took four months longer. In general, the drop of antibody level was faster in men than in women. The authors did not succeed in establishing why antibodies disappeared from the sera of some women and men considerably more rapidly than from those of others, but it can be noted that the rate at which antibodies disappeared did not depend upon the clinical form of trichomoniasis. However, the results confirm that specific antibodies are present only in the sera of people suffering or having recently suffered from urogenital trichomoniasis. On the basis of all the results, the Estonian workers concluded that the complement-fixation reaction constitutes a useful tool in diagnostic and epidemiologic studies of human urogential trichomoniasis, provided that a number of antigenic types of T. vaginalis prevalent in a given

locality are employed simultaneously as antigens. Further studies would however be needed to reconcile the results of Korte, Hoffmann and his collaborators, and the Estonian workers.

A.12.2 Agglutination

The earlier reports on the employment of agglutination for diagnosis of T. vaginalis include data that throw much doubt on the applicability of this method for diagnostic purposes. Honigberg (1978b) reported that, even today, many investigators (unpublished data of various workers) have had great difficulty in using agglutination as an effective diagnostic tool, even with parasites isolated from patients whose sera were tested. The presence of natural antibodies against T. vaginalis or of antigenic differences among isolates are two of several factors which render this test unreliable. For instance, some of the titres obtained with sera of infected patients were below those generally considered as due to natural antibodies and therefore nonspecific. In other instances, only a relatively small percentage of sera from patients found positive for trichomonads on microscopic examination had significant agglutination titres.

Trussell (1946) was among the first to investigate agglutination techniques for the diagnosis of

human urogential trichomoniasis. Results of this work were negative and Trussell (1946) recorded only negligible and probably nonspecific titres (mostly 1:10 and 1:20) in a very small number of sera from 182 women (90 pregnant and 92 syphilitic - groups typically characterized by a high incidence of infection with T. vaginalis). Experimentally, Trussell (1946) observed titres ranging from 1:640 to 1:5,120 with sera of rabbits immunized against T. vaginalis.

Using the agglutination reaction, Tatsuki (1957) examined sera of 32 female patients harbouring T. vaginalis. He found 84.5% gave agglutination reaction with titres ranging from 1:16 to 1:512 and averaging 1:124. He also examined sera from 17 parasite-free women and obtained positive agglutination reaction in 52% with much lower titres ranging from 1:8 to 1:64 and averaging 1:30. Among 19 boys, only two (10.5%) were found to have even low agglutinin titres. In the light of this work, Tatsuki (1957) considered the results obtained with the parasite-free women as genuine and reflecting in some instances the presence of infection that might have been overlooked on direct examination, and in others a history of past infection. He also considered titres which exceeded 1:4 as significant; however, it seems to us that this conclusion contradicts most of the recent investigators who have been inclined to reject as nonspecific

all low titres, even in some instances up to 1:160.

Arai (1959) recorded agglutination titres reaching 1:10,240 with anti-T. vaginalis rabbit sera and between 1:10,240 to 1:20,480 with immune mouse sera. He also examined sera of 27 persons infected with T. vaginalis and he found titres ranging from 1:6 to 1:32. However he claimed that in unsuccessfully treated cases, the agglutinating capacity of the serum was unaltered, but successful medication was followed by a gradual drop in titre.

Using a formalinized antigen, Stepkowski and Bartoszewski (1959) obtained a positive agglutination reaction with sera from only 60% of persons found to harbour T. vaginalis on direct examinations. In the light of their results, they were convinced of the specificity of the agglutination reaction, but considered this test to be of only limited practical use in the diagnosis of human urogenital trichomoniasis.

In the use of the direct agglutination reaction, it is again the Estonian group of Teras and his colleagues (1966a and b) who have been most conspicuously successful. In all experiments, the Estonian workers employed axenically grown, living trichomonads washed with and resuspended in physiological saline. To establish the level of dependence of agglutination upon the antigenic type of T. vaginalis used in the reaction, the types of strains from 99 patients

were ascertained by reacting them with sera from rabbits immunized by the four basic antigentic types defined by these workers: TR, TRT, TN and TLR (Teras et al., 1966a; Jaakmees, 1965, quoted by Honigberg, 1970). Having ascertained the antigenic identities of the strains infecting the 99 patients, Jaakmees (1965, cited from Honigberg, 1970) employed trichomonads of the same types in agglutination tests. The results of tests (from Hongiberg, 1970) are summarized in Table 3.

The highest titre found in sera from uninfected controls was 1:160 (Teras et al., 1966a) and twice this was regarded as diagnostic (see Table 3). In Table 3, significantly fewer clearly positive reactions were obtained with heterologous antigenic types and fewer positive cases would have been found by serodiagnosis if only one antigenic type was used in the agglutination test. As in the complement-fixation reaction, the Estonian workers pointed out that agglutination method was not useful for the identification of the type of the flagellate strain infecting the donor of a given serum. In many instances the agglutination titre might be equally high in a homologous and a heterologous system.

In a major study, Nigesen (1966, quoted by Honigberg, 1970) employed the agglutination reaction in a study of 583 human sera. One group included 256 women and 130 men known to harbour T. vaginalis; the

Table 3: Dependence of the agglutination reaction on the antigenic type of *T. vaginalis* employed in tests with sera from infected persons*

Sera from persons infected with type:	No. of sera	Agglutination titre 1:320 or over with antigens					
		TR	TRT	TN	TLR	Homologous	All
TR	43	43	30	27	24	43	15
TRT	29	15	28	19	11	28	6
TN	14	7	8	13	4	13	2
TLR	13	7	8	9	13	13	3
Total	99	72	74	68	52	97	26

* After Jaakmees, 1965, (quoted by Honigberg, 1970).

second consisted of 45 persons who, although negative for trichomonads, were sexual partners of infected individuals; the third, a control group, was represented by 152 individuals found to be free of the parasites on repeated examination. Organisms of the four antigenic types recognized by the Estonian workers were used to test each serum. Table 4, quoted from Honigberg (1970), summarises the results obtained with the sera from patients infected with T. vaginalis. It is evident that over 94% of sera from both infected men and women gave clearly positive agglutination reactions, whose titres always exceeded 1:320.

There appears, however to be little, if any, correlation between the agglutination titres of the sera and the clinical manifestations observed in the donors of these sera (Teras et al., 1966b; Honigberg, 1970). Sera from 7 female and 14 male patients gave non-significant titres in the agglutination reactions (Table 4). Discussing this, Nigesen (1966, quoted by Honigberg, 1970) suggested that they might reflect an immunologic difference between the infecting parasites and those employed in the agglutination tests, or that they represented a relatively recent acquisition of the parasites which had not yet stimulated the production of agglutinin. The study of sera from 45 parasite-free individuals whose sexual partners suffered from urogenital trichomoniasis also showed the presence of specific agglutinins,

Table 4: Agglutination reaction with sera from patients showing different clinical manifestations of urogenital trichomoniasis*

Clinical manifestation	Women			Men		
	No.	Titre over 1:320	Titre 1:200 to 1:240	No.	Titre over 1:320	Titre 1:200 to 1:240
Acute or subacute	127	122	5	20	20	0
Chronic	115	113	2	70	65	5
None**	14	14	0	40	31	9
Total	256	249	7	130	116	14

* After Nigesen, 1966 (quoted from Honigberg, 1970)

** "Latent" trichomoniasis.

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None**	14	14	0	40	31	9
Total	256	249	7	130	116	14

* After Nigesen, 1966 (quoted from Honigberg, 1970)

** "Latent" trichomoniasis.

with titres at least twice as high as those expected in normal sera. Nigesen (1966, quoted by Honigberg, 1970) postulated that such individuals could have had cryptic infections limited to the higher regions of the urogenital tract, pointing out that the detection of trichomonads in such sites is difficult, even by culture methods. This hypothesis is at variance with those expressed by other investigators (Coutts and Silva-Inzunza, 1957; Teras, 1959, 1964; Jaakmees, 1965; both Teras and Jaakmees are quoted by Honigberg, 1970) in regard to seropositive but apparently parasite-free individuals. However, like the results obtained with the sera of women and men harbouring T. vaginalis, those obtained with the sera of the parasite-free sexual partners of infected individuals depended to a significant degree upon the antigenic types of the trichomonads employed in the agglutination reaction. As an important part of this study of agglutination reactions in trichomoniasis, Nigesen (1966, quoted by Honigberg, 1970) examined changes in the specific antibody level in the sera of 24 women and 17 men who underwent treatment with metronidazole. The sera were examined for the presence and level of specific agglutinating antibodies before and for a year and a half after the course of treatment. It was ascertained by a series of microscopic examinations and by repeated attempts at cultivation that in all cases the trichomonads disappeared not later than on the fourth

day of treatment. There was a progressive drop in the agglutination titres during the first two months following treatment, but even after 6 months the reaction remained clearly positive in about 50% of the female patients and less definitely so in the remaining women. In most female patients the agglutinin titres dropped to normal within 9 to 12 months, and in all cases antibody was undetectable after 16 months following the administration of metronidazone. A similar situation was noted in men, except that in many cases normal serum titres were reached within six months. The rate of drop of the titres did not seem to show any correlation with the clinical form of trichomoniasis nor with the original pretreatment antibody levels.

A.12.3 Indirect haemagglutination

There have been relatively few papers published on serodiagnosis using a haemagglutination test. McEntegart (1952) employed the technique in serodiagnosis of human urogenital trichomoniasis. Antigen was prepared by the formamide method (Fuller, 1938) from 6 strains of T. vaginalis isolated from patients. Having established the optimum conditions, sera from 50 normal men and 50 normal women (donated blood), from 13 normal children (7 males and 6 females) and 50 women infected with T. vaginalis (attending a

Venereal Diseases Department and with or without clinical manifestations) were employed in the experiments. All the 163 sera were inactivated at 56°C for 30 minutes and were absorbed with sheep red cells prior to testing to eliminate the possibility of non-specific haemagglutination. The results of the experiments are summarized in Table 5.

In the light of his results, McEntegart (1952) admitted that the haemagglutination reaction observed with low dilutions of sera used as controls might have been dependent upon cross-reactions with the other two trichomonads, T. tenax and Pentatrichomonas hominis or else might reflect actual infections. Although Tokura (1935) observed that the three trichomonads which may occur in the human being, i.e. P. hominis, T. tenax and T. vaginalis, gave serologically distinct reactions when tested against immune rabbit serum, the first suggestion cannot be accepted in the absence of actual demonstration of such antigens in the host organism. The other suggestion is plausible, since the normal control group was not examined for the presence of the urogenital trichomonads. Since some positive reactions were found using the sera of children at 1:5 and 1:10 dilutions, McEntegart was inclined to consider such reactions as nonspecific. The difficulty in interpreting these experiments is partly due to the relatively small samples and the

Table 5: Results of haemagglutination test with *Trichomonas vaginalis**

	Serum from: Normal men	Normal women	Normal children	Parasitized women
Total number	50	50	13	50
Positive at dilutions:				
1:5**	6	3	4	2
1:10**	10	5	3	5
1:20	3	3	0	15
1:40	0	0	0	12
1:80	0	0	0	10
1:160	0	0	0	2
1:250	0	0	0	3
Total positive***	19(3)	11(3)	7(0)	49(42)
Percent***	38(6)	22(6)	54(0)	98(84)
Total negative***	31(47)	39(47)	6(13)	1(8)
Percent***	62(94)	78(94)	46(100)	2(16)

* After McEntegart, (1952).

** Probably nonspecific reactions.

*** Numbers and percentages in parentheses by considering positive reactions at 1:5 and 1:10 dilutions as nonspecific.

rather inadequate control group. Nevertheless, it is quite likely that, as he says: "possibly improvements in the antigen employed ... would lead to an increase in the titre obtained with positive sera and ... make the distinction between true and false positive easier". Undoubtedly the improvement of the antigen would have to include also the employment of several antigenic types (Honigberg, 1970).

Using haemagglutination, Lancely and McEntegart (1953) examined the sera of five male human volunteers experimentally infected with bacteria-free cultures of T. vaginalis. Three cases definitely became infected and all showed varying degrees of urethritis, but none produced antibody detactable by indirect haemagglutination. In the light of the subsequent findings of Hoffmann (1966b) and also as reported by Honigberg (1970), one must view with caution the conclusion reached by Lancely and McEntegart (1953) that the negative results obtained by them might have been expected, because in bulls infected with Tritrichomonas foetus, serological response is elicited only in unusually severe infections. However, the serologic response is in many aspects different in Tr. foetus and T. vaginalis infections.

More recently, attempts to use the haemagglutination method in the serodiagnosis of

trichomoniasis were reported by Hoffmann (1966b). He employed a formamide extract (Fuller, 1938) of five freshly isolated trichomonad strains (belonging to as many antigenic types) for sensitisation of sheep erythrocytes. Sera from 380 persons infected with T. vaginalis and from 1242 individuals chosen at random (controls) were tested. Agglutination by a 1:10 dilution of sera was considered as weak positive, while reaction at 1:20 or higher dilutions of sera were considered as positive. Table 6 and Table 7 summarise the results obtained.

In general, the titres obtained with sera from infected women were higher than those from infected men. In both sexes, the highest percentage of positive results was observed in patients with chronic infections, while the test appeared to be least effective with sera of individuals showing symptoms associated with acute trichomoniasis. The percentage of positive sera from the control group was found to be much lower than that from infected persons. Hoffmann assumed that some or all of the donors from the random group with positive sera were either infected with T. vaginalis or had a history of previous infections with this parasite. Not only this suggestion in agreement with other investigators but also it is supported by the results of examining urogenital secretions of 126 women and 94 men from the random group.

Table 6: Haemagglutination titres of sera from persons infected by Trichomonas vaginalis*

Sex	Clinical manifestations	No.	Serologic reactions weak positive** No.	%	positive*** No.	%	mean titre
Female	Acute	16	6	37.5	8	50.0	1:17.7
	Chronic	93	1	1.1	92	98.9	1:68.7
	None	77	8	10.4	68	88.3	1:25.4
Totals		186	15	8.1	168	90.3	1:46.4
Male	Acute	24	12	50.0	7	29.2	1:10.0
	Chronic	74	10	13.5	59	79.7	1:48.3
	None	96	46	47.8	40	41.8	1:18.3
Totals		194	68	35.0	103	54.6	1:28.8

* After Hoffmann, (1966b).

** Weak positive: reaction at dilution 1:10.

*** Positive: reaction at dilution 1:20 or higher.

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	None	77	8	10.4	68	88.3	1:25.4
Totals		186	15	8.1	168	90.3	1:46.4
Male	Acute	24	12	50.0	7	29.2	1:10.0
	Chronic	74	10	13.5	59	79.7	1:48.3
	None	96	46	47.8	40	41.8	1:18.3
Totals		194	68	35.0	103	54.6	1:28.8

* After Hoffmann, (1966b).

** Weak positive: reaction at dilution 1:10.

*** Positive: reaction at dilution 1:20 or higher.

Table 7: Haemagglutination titres of sera from a group of persons chosen at random*

Sex	Age	No.	Serologic Reactions				Mean titre
			Weak positive**		Positive***		
			No.	%	No.	%	
Female	under 15 years	83	30	36.1	6	7.3	1: 4.4
	over 15 years	540	204	37.8	233	43.2	1:15.6
Totals		623	234	37.5	239	38.4	1:14.0
Male	under 15 years	96	43	44.8	0	0.0	1: 3.3
	over 15 years	523	216	41.3	125	23.9	1: 9.6
Totals		619	259	41.8	125	20.2	1: 8.6

*After Hoffmann, (1966b).

** Weak positive: reaction at dilution 1:10.

*** Positive: reaction at dilution 1:20 or higher.

Thirty eight women and eleven men were found to be infected. On the basis of his results, Hoffmann (1966b) concluded that the passive haemagglutination method combining relative simplicity with high levels of sensitivity and specificity, holds promise as a tool in serodiagnostic and epidemiologic studies.

Although Uminski et al. (1973) found complement-fixation and haemagglutination methods to be about equally effective in the diagnosis of T. vaginalis infections, Hoffman's (1966b) results suggest that the latter technique is somewhat superior in terms of positive correlation between the serologic findings and those obtained by microscopic examinations and the culture methods.

A.12.4 Fluorescent Antibody

A.12.4.1 Direct fluorescent antibody

McEntegart et al. (1958) were probably the first to apply fluorescent antibody techniques for finding organisms in cases of trichomoniasis. Anti-T. vaginalis serum was prepared in rabbits as described by McEntegart (1956). The globulin fraction of the serum was separated and conjugated to fluorescein isothiocyanate. Using this technique, McEntegart et al. (1958) succeeded in differentiating T. vaginalis from Tr. foetus, and were able to demonstrate distinct strains

of T. vaginalis. They suggested that greater purification would be needed to overcome the nonspecific staining of leucocytes if vaginal smears were employed.

In a short abstract, Hays and Kotcher (1960) indicated that the efficiency of the direct fluorescent antibody technique was comparable to that of the cultivation method; of 225 specimens of vaginal secretions, 39.6% were diagnosed as positive for the urogenital trichomonad by fluorescent antibody technique and 40% by cultivation in sodium thioglycollate medium. Both methods were found superior to wet mounts of fresh material (33.3%) and Papanicolaou-stained smears (13%).

A.12.4.2 Indirect Fluorescent Antibody

According to the recent report of Kramar and Kucera(1966), the indirect fluorescent antibody technique is applicable to the diagnosis of trichomonad infections. Formalinized trichomonads derived from axenic cultures and fluorescein isothiocyanate-conjugated rabbit anti-human gamma globulin were employed in the fluorescent antibody test. They examined sera from 17 female patients with positive microscopic findings of T. vaginalis (all were acute cases of over two months' duration) and two male patients with positive microscopic and culture finding. The control

sera came from 5 infants under one year of age and from 2 women and two men, found negative for T. vaginalis on direct examination. All sera from cases infected with T. vaginalis showed positive reaction, while sera from negative donors showed negative reaction. Apparently the method has not been tried again.

Although potentially very promising (Honigberg, 1970), the fluorescent antibody methods have not been explored widely as tools in diagnosis and epidemiologic studies of T. vaginalis. Certainly this method has proved most useful in studies of amoebiasis (Goldman and Gleason, 1962; Goldman and Canon, 1967), malaria (Tobie and Coatney, 1961; Kuvin et al., 1962) and toxoplasmosis (Fletcher, 1965; Walton et al., 1966) and many other protozoal infections.

A.12.5 Skin Test (Delayed Hypersensitivity Reaction (DTHR))

Attempts to develop a skin test for trichomoniasis occupied the attention of many investigators. Among the early attempts were those of Wendlberger (1936) who reported inconclusive results with an alcohol-extracted antigen, and Trussell (1947) who used whole bacteria-free organisms for intradermal inoculation. Nearly all persons tested showed

indefinite reaction which were regarded as non-specific.

Experimentally, Lancely (1958) was unable to distinguish between skin reactions obtained by intradermal inoculation of T. vaginalis or of the control (sterile medium) into rabbits that had been immunized with the organism. On the other hand, Adler and Sadowsky (1947), employing a phenol-extracted antigen, examined 43 cases shown to harbour the urogenital trichomonads and 59 cases in which T. vaginalis was not found on microscopic examination. A reaction 1 to 2 cm in diameter and appearing within 48 hours after intradermal inoculation was considered positive. Among 43 patients shown to harbour T. vaginalis, 35 (81%) gave a clearly positive skin reaction, 2 were negative, and 6 showed an indefinite reaction. Adler and Sadowsky (1947) suggested that positive skin reactions develop because antigens of T. vaginalis are absorbed through the wall of the vagina in amounts sufficient to produce specific sensitization. However, there appeared to be no correlation between the intensity of the skin reaction and the clinical manifestations, but the most intense responses were associated with infections refractory to treatment. On the other hand, the results of the controls (59 cases in which T. vaginalis was not found on microscopic examination) were 45 negative, 7 indefinite, and 7 positive in skin reactions. One of the persons

belonging to the last group was found to harbour T. vaginalis on a second direct examination, another was diagnosed again as negative for the parasites, and the remaining five could not be traced for second examination. Inasmuch as a higher percentage of T. vaginalis infection was found by direct examination than by the skin test, Adler and Sadowksy (1947) admitted that the skin test is not to be recommended for diagnostic purposes.

Aburel et al. (1963) surveyed 263 women for the presence of T. vaginalis by both intradermal testing and direct microscopic examination. Among the persons found to harbour the parasite 82% gave positive skin reactions (1 cm in diameter appearing 24 hours after inoculation). The author suggested three possible reasons for the failure of the remainder to react. Firstly, the infecting parasites belonged to different antigenic types than those employed in the test; secondly, the infection could have been relatively recent; or thirdly, the lack of the skin reaction might have been dependent upon anergy of some individuals. If this is true, it might be useful in the future to add more to fundamental knowledge of the immune response to this parasite.

Apart from the above explanation, twenty-five per cent of women found negative for T. vaginalis by direct microscopic examination (and in some instances

by culture) gave a positive skin reaction. Again, Aburel et al. (1963) was in agreement with Adler and Sadowsky (1947) in suggesting that some of those individuals might have had a previous infection. In conclusion the authors suggested that, "Intradermal testing may ... considerably reduce the number of direct examinations and cultures required for diagnosis of genitourinary trichomoniasis".

Sinelnikova (1961, quoted by Hongiberg, 1970 and 1978b) studied the allergic skin reaction in urogenital trichomoniasis in man and its diagnostic significance. Three different antigens were employed. The first was an extract obtained by suspending dried, powdered trichomonads in physiological saline; the second was hot acid hydrolysate of the parasites and the third a "corpuscular antigen" prepared according to the method of Anina-Rachenko (1959, quoted by Jaakmees and Teras, 1966 and Hongiberg, 1970). Positive skin reactions (1 - 2 cm after 24 hours) were found in 61% of women known to harbour T. vaginalis when she employed the first antigen and in 41% of the same group on employing the second antigen. On employing the third antigen, 77% of infected women gave positive reactions. Trichomonas-free persons were found negative by skin test when tested with all three antigen preparations.

More recently, Jaakmees and Teras (1966)

and Jaakmees (1965, quoted by Honigberg, 1970) investigated the skin test and examined 324 persons of both sexes (209 infected and 115 T. vaginalis-free on direct examination or by culture). They used as antigen an "aqueous lysate" of the four different serotypes recognized by the Estonian investigators. Among 40 Trichomonas-free sexual partners of women suffering from trichomoniasis, positives reactions (greater than 5 mm diameter after 24 - 48 hours) were found in 3, weakly positive in 9, and negative in 28 men. Of five Trichomonas-free sexual partners of the men suffering from trichomoniasis, one women gave a positive and another a weakly positive reaction whereas the remaining three gave negative reactions with the four antigens. Jaakmees and Teras (1966) and Jaakmees (1965, quoted by Honigberg, 1978b) observed that the highest number of positive reactions were recorded from patients suffering from acute or subacute trichomoniasis, and the intradermal test proved negative more frequently in women suffering from chronic or latent trichomoniasis. Subsequently using "corpuscular antigen" (cell-free homogenates of trichomonads suspended in 0.85% saline with 0.5 phenol) the Estonian investigators (Jaakmees and Teras, 1966; Jaakmees, 1965, quoted by Hongiberg, 1978b) observed a positive skin reaction in all (21) women with T. vaginalis; not a single positive reaction was

recorded from 40 (20 women and 20 men) parasite-free individuals. It seems that the demonstration of higher numbers of positive reactions might be due to the use of the "corpuscular antigen"; in addition a smaller erythematous area was regarded as a positive reaction than was usual in earlier work.

No correlation could be established between the presence and/or intensity of the intradermal reactions in patients who harboured T. vaginalis and the agglutination or complement-fixing titres of the sera from these persons. In fact, completely negative results with the skin test were observed in individuals whose sera showed high levels of agglutinating and complement-fixing antibodies.

In the light of their results, the Estonian investigators were of the opinion that further standardization of the method would be needed before intradermal tests could be considered for practical use.

It seems clear from the available data that the presence of the urogenital trichomonad in man stimulates the production of specific reactivity in a proportion of patients. In the light of all the results summarized above, it seems that at present the skin test does not appear to provide any significant advantages over direct examination in routine diagnosis. Perhaps further improvements will render the test effective in demonstrating cryptic infections and in tracing infected sexual partners of parasite-free individuals.

A.12.6 Local antibody

Ackers et al. (1975) drew attention to the lack of success of previous workers in demonstrating local, vaginal antibody to T. vaginalis by direct agglutination and indrect immunofluorescence. However, by means of a radioimmune assay IgA antibody to the urogenital trichomonad was found in the vaginal secretion of 76% of 29 infected, and 42% of 19 apparently uninfected women; however, the average number of counts was twice as great in secretions from the former than from the latter group. No correlation was found between the presence or absence of antibody and severity of inflammation, duration of symptoms, the use of oral contraceptives or accompanying sexually transmitted infections. There was, however, a suggestion of some possible association between the presence of local antibodies and reduced parasite counts. The existence of such an association appears to be supported by the observations of Chipperfield and Evans (1972), who with the aid of the direct fluorescent antibody method, noted an increase in IgA-, IgG- and IgM-plasma cells in the lamina propria of the endocervix of women infected with T. vaginalis, Neisseria gonorrhoea and Candida albicans.

Although the percentage of women examined by Ackers et al. (1975) whose vaginal secretions contained

antibodies to T. vaginalis was higher among the individuals harbouring this parasite than among Trichomonas-free persons, the presence of these antibodies cannot, at this time, be considered diagnostic. There was no evidence that any of the women negative for T. vaginalis but positive for local antibodies had histories of previous infections; nevertheless such a possibility certainly cannot be dismissed. Much more work will need to be done before any definite conclusion can be reached about the true incidence and significance of antitrichomonal IgA antibodies found in vaginal secretions.

More recently, Ackers et al. (1978) employed a similar assay to test 37 samples of semen and urine from male contacts of women with proved Trichomonas infection. Antitrichomonal antibodies were found in only 2 out of 8 samples positive for T. vaginalis on culture. The authors suggested that the lack, in men, of a vigorous immune response to such a scanty and non-invasive parasite as T. vaginalis is perhaps not surprising, especially as it is not clear how long the organisms remain in the male urethra after infection. It is quite possible that the repeated passage of urine mechanically removes the parasite within a few days. They concluded that the asymptomatic nature and low parasite numbers commonly described in infections in men is thus unlikely to be due to a vigorous local immune response.

Although the radioimmune assay employed by Ackers et al. (1975 and 1978) is more sensitive than many other assays modifications would be necessary if it were to become useful in practice, and the method seems more likely to be of use in studying the fundamental nature of the response to intra-vaginal antigens.

A.13 Objective of the Present Work

A.13.1 Characterization of T. vaginalis

When a parasite is obnoxious to man or his domestic animals, there is a fundamental need to identify the organism involved not for slotting into a taxonomic niche, but to help the clinician predict the outcome of the infection and decide how to effect a cure. Godfrey (1978) has pointed out that because of our incomplete knowledge of the occurrence, dissemination and control of many parasitic diseases, biochemical techniques for the identification of isolates of parasitic protozoa, such as Leishmania, Trypanosoma, Entamoeba, Plasmodium and others have become essential to attempts to resolve difficulties in understanding the epidemiology of parasitic diseases. This suggestion was supported by Honigberg (1978b) who wrote that in T. vaginalis "there can be no doubt, however that chemical analysis constitutes a fruitful area for future studies. The inability to differentiate

easily between strains of T. vaginalis has been considerable hindrance to research into many aspects of trichomoniasis especially its epidemiology and immunology".

Characters other than morphological ones, have been used in attempts to distinguish between morphologically similar protozoa. Lumsden (1974b) divided the characters used in taxonomy and identification of parasitic protozoa into two groups:

I. Intrinsic characters, relating to the organism itself.

Morphology: By light microscope
By electron microscope

Chemical structure:
DNA buoyant density
Isoenzyme constitution

Immunological effect:
Humoral response
Cell-mediated response
Protection

II. Extrinsic characters, relating to the response of other components to the invasion of the organisms.

Behaviour in laboratory hosts
Behaviour in insect vectors
Behaviour in culture
Clinical outcome in man.

A.13.2 Differences among isolates of T. vaginalis

A.13.2.1 Morphology

Observation by previous workers on T. vaginalis showed that individual variations can be seen among members of single populations of T. vaginalis. The morphology of T. vaginalis has been widely studied. Using light microscope, Fienberg (1954) described club-like structure on the terminal one-fourth to one-third of one of the four free flagella in 3 different strains of T. vaginalis; he claimed that this structure varies between strains and between individuals in the same strains. The size of T. vaginalis varies from one patient to another and even among populations from the same patient at various stages of infection. Because of these observations, Honigberg (1978b) pointed out the need for great caution in attempts to differentiate species of Trichomonadinae on the basis of light microscope studies and even more in attempts at differentiating strains within a single species. The electron microscope studies (e.g. Nielsen, 1975) on T. vaginalis gave no direct information on the morphological differences between virulent and avirulent strains.

A.13.2.2 Pathogenicity

Teras (1966) claimed that there was reason to believe that the species of trichomonads parasitizing the genito-urinary tract consist of different types (possibly even different subspecies) on which depended the pathology and clinical course of trichomoniasis. The Estonian investigators (e.g. Teras, 1966; Teras and Roigas, 1966; Laan, 1966) using intraperitoneal inoculation in mice, could detect differences in the pathogenicity of several strains of T. vaginalis on the basis of gross and histopathological characteristics of the lesions, time necessary for their development and length of time needed for the parasite to kill the mice. They distributed T. vaginalis strains into 3 groups:

(a) Group (1), strains of high pathogenicity: characterized by frequent invasions to abdominal organs of the experimental animals with rapid pathologic changes leading to high mortality within 5 to 7 days.

(b) Group (2), strains of medium pathogenicity: this group is characterized by weaker invading ability and lower pathogenicity to internal organs.

(c) Group (3), strains of low pathogenicity: infection is characterized by the presence

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(c) Group (3), strains of low pathogenicity: infection is characterized by the presence

> of small amounts of exudate; invasion of internal organs is very rare.

According to these workers, the strains of high or medium pathogenicity have been isolated mainly from patients suffering from acute or subacute trichomoniasis; and the strains of low pathogenicity from patients suffering from chronic and latent trichomoniasis.

Other measurements of the virulence of T. vaginalis in mice (e.g. Ivey and Hall, 1964; Honigberg, 1961; Kulda et al., 1970; Honigberg et al., 1966) also reveal differences between individual strains. These authors, using subcutaneous inoculation in mice of isolates of T. vaginalis, estimated differences in pathogenicity by measuring the mean volumes of six-day lesions.

Kulda and Petru (1966) and Ivey and Hall (1964) using subcutaneous test of Honigberg (1961) confirmed that within T. vaginalis species there are strains exhibiting different degrees of virulence. These authors also carried out parallel intraperitoneal test, and found that the results were considerably different from those shown by the subcutaneous test. However, they felt that the results of subcutaneous tests were more reliable in indicating the relative pathogenesis of different strains of T. vaginalis. Apparently though the results of this

work have been obtained with very few strains, most of which have been maintained in culture for many years; moreover, large numbers of animals are necessary to obtain valid results. At present not enough has been done to evaluate the true value of this test.

A.13.2.3 Biochemistry

Although during the last few years some differences between strains of T. vaginalis have been defined, nevertheless very scanty data are available concerning the intraspecific variation in the biochemical activity of trichomonads and the relationship of these varieties to other biological properties. In fact little information has been published recently on biochemical aspects of T. vaginalis other than those of carbohydrate metabolism (Honigberg, 1978b; Shorb, 1964). Teras et al. (1973) determined the hexokinase activity in a cell-free homogenate and found that all the 25 strains of T. vaginalis under investigation phosphorylated glucose but with varying intensity. The strains could be divided into groups with low, medium and high hexokinase activity, and they found that the strains of T. vaginalis that rapidly phosphorylated glucose were more virulent than those with low hexokinase activity although neither virulence nor the enzyme activity depended on the serotype of T. vaginalis. The ability of T. vaginalis

strains to ferment maltose, lactose, sucrose and mannitol was also found to be very variable (Laan, 1966; Teras, 1961 and 1966). According to gas production and type of sugar used, Teras (1961) divided T. vaginalis strains into three groups. On examining the relationship between fermentative activity and virulence among different T. vaginalis strains, Laan (1966) found that the fermentative activity of the strains of high virulence was comparatively less than that of the strains of medium and low virulence. This result is apparently in conflict with those of Teras et al. (1973). However, Honigberg (1978b) pointed out that the reported anaerobic fermentation products of T. vaginalis are very varied, probably due to strain differences or differences in experimental procedures; the variability of metabolic products of T. vaginalis, which exceeds that found with other protozoa, renders comparison exceptionally difficult (Danforth, 1967). Attempts to interpret the biochemical attributes of urogenital trichomonad of man on the basis of data derived from studies of the more easily grown species (especially Tritrichomonas foetus) are probably invalid (Honigberg, 1978b).

Finally, by means of disc electrophoresis of protein fractions of ten isolates of T. vaginalis Andreeva et al. (1976) were able to distribute the strains of T. vaginalis by twos into five groups.

A.13.2.4 Immunology

Teras (1966) assumed that strains of T. vaginalis that are of different pathogenicity must also differ in their antigenic structure. Immunological studies certainly showed that antigenic differences among the strains of T. vaginalis existed (e.g. Schoenherr, 1956; Magara, 1957; Amino, 1958; Lancely, 1958; Teras, 1965, 1966, 1961; Kott and Adler, 1961; Hoffmann and Gorczynski, 1964; Teras et al., 1966; Laan, 1966); the presence of different antigenic types was demonstrated experimentally by precipitation, agglutination, complement-fixation, haemagglutination. Using these tests, many investigators were able to show at least three (e.g. Schoenherr, 1956; Magara, 1957; Lancely, 1958; Hoffmann and Gorczynski, 1964) up to eight (e.g. Kott and Adler, 1961) discrete antigenic types. These results were found to depend largely on the strains of T. vaginalis used as antigen.

Differences among strains of T. vaginalis were demonstrable also in the results obtained by serological examinations of the sera of patients suffering from trichomoniasis. Using a single test (e.g. agglutination) considerable differences in titres were found among many cases examined, and the results were found to depend on the serotype of T. vaginalis used as antigen. However no real correlation of serotype with pathogenicity has been demonstrated.

Kott and Adler (1961) and Hoffmann and Gorczynski (1964) suggested the occurrence of several antigenic types of T. vaginalis within a quite limited geographical area; conversely Teras (1966) proved that the antigenic structure of the VP strain from Czechoslovakia was identical with the TLR type from Estonia, and that the antigenic structure of the LZ strain from Hungary was identical with the TR strain from Estonia.

A.13.2.5 Drug sensitivity

It is possible that differences in sensitivity to various trichomonocidal drugs, particularly metronidazole (Flagyl) may reflect differences between the strains of T. vaginalis used in the test (Ings, 1973; Jennison et al., 1961; Squires and McFadzean, 1962; De Carneri, 1969; Benzat and Guillaume, 1971; Samuels, 1961; Samuels and Stouder, 1962).

A.13.2.6 Growth rate

Generation time was found to vary among strains of T. vaginalis. Three strains of T. vaginalis showed an inverse relationship between their growth rates and pathogenicity, as determined by the

subcutaneous mouse assay (Honigberg, 1961). Kulda et al. (1970) compared the generation times of nine T. vaginalis strains and their pathogenicity as judged by the subcutaneous mouse assay. Relatively short generation times were characteristic of all the mild strains and those of moderate pathogenicity. On the other hand, the generation time of highly pathogenic parasites was long.

A.13.2.7 Isoenzymes

It is now accepted that the intraspecific differences are the result of inherited variations, developed during the course of evolution, in the structures (and amounts) of the proteins of the species concerned (Bagester and Parr, 1973). Clearly if analogous proteins in different strains of a species could be characterized in some method (such as electrophoresis) which can reveal small structural differences, biochemical classification of the different strains might be possible. Some progress along these lines has already been made in the field of protozoology. For instance, different strains as well as different species of Plasmodium were recently reported to differ, to a lesser and a greater degree respectively, in electrophoretic characteristics (Carter and Voller, 1973). Godfrey (19[illegible]) claimed

that the biochemical methods could now offer objective definitions of subspecies. One such method, the characterization of multiple enzyme forms (isoenzymes) is very suitable for characterising strains amongst Trypanosoma species.

Enzymes are large protein molecules whose primary structures are genetically determined, and each performs a specific biochemical function in the cell. Many enzymes have the same function in many kinds of organisms, yet differ from each other in molecular structure, perhaps only slightly. The name isoenzyme (or isozyme) (Markert and Moller, 1959) is used to describe an enzyme with multiple molecular forms. The isoenzymes may have the same catalytic action, but differ in their kinetics, and they may occur together in the same cell. Such a difference may be associated with a dissimilarity in the net electric charge on the molecule, and may be detected by comparing electrophoretic mobilities on a gel (Markert and Moller, 1959; Markert, 1975; Wilkinson, 1969; Harris and Hopkins, 1976). The gel can also separate molecules by virtue of its ability to act as a molecular sieve. The position of the enzyme is shown by a specific staining reaction.

Isoenzymes have been detected among many different enzymes. Since its development, enzyme electrophoresis has provided a means of indicating

molecular differences among specific products of organism population and has had widespread application particularly in the field of human genetics (Harris and Hopkins, 1976; Harris, 1966).

Among protozoa, the isoenzyme technique, first applied to the soluble enzymes of trypanosomes by Kilgour and Godfrey (1973) and Bagester and Parr (1973), has become widely used in characterization of parasitic protozoa, e.g. Leishmania (Kilgour et al., 1974; Kilgour, 1976; Al Taqui and Evans, 1978; Gardner and Howells, 1972; Gardner et al., 1974; Ebert, 1973 and 1974), Entamoeba (Reeves et al., 1968; Montalvo et al., 1968; Sargeaunt et al., 1978 and 1978 and 1979; Williams et al., 1978); Naegleria (Warhurst et al., 1978), Plasmodium (Carter and McGregor, 1973; Carter and Voller, 1973 and 1979), Trypanosoma (Miles et al., 1977 and 1978; Godfrey, 1975, 1977 and 1978; Godfrey and Kilgour, 1976; Kilgour et al., 1973 and 1975; Kilgour, 1976; Bagester and Parr, 1973; Gibson, 1977; Gibson et al., 1978 and 1978).

The aim of this work was to apply this and other techniques to the study of T. vaginalis and to seek correlation between biochemical markers and biological behaviour. Isolates of T. vaginalis from patients at James Pringle House (Venereal Diseases Clinic, Middlesex Hospital, London, WC1) have been compared with each other by means of isoenzyme

electrophoresis; in addition to the human isolates, we have obtained a small number of less common Trichomonadinae to compare with the human vaginal organism. These included monkey faecal parasites (Trichomitus spp.), mouse and hamster-virulent strains of T. vaginalis and authentic Pentatrichomonas hominis. The technique of starch gel isoenzyme electrophoresis was chosen for this work because it requires only a small amount of material; eight to twelve samples can be directly compared, and the results are rapidly available.

PART II

MATERIALS AND METHODS

B. MATERIALS AND METHODS

B.1 Sources of Our Samples

(1) Trichomonas vaginalis from James Pringle House (the sexually-transmitted diseases clinic of the Middlesex Hospital, London, W.C.1).

(2) Intestinal Trichomonas from monkeys at London Zoo, kindly provided by Mr Peter Sargeaunt and Mr John Williams.

(3) Trichomonas vaginalis from Italy, kindly provided by Professor Cappuccinelli, University of Torino, Institute of Microbiology, Italy.

(4) Hamsters infected intravaginally with Trichomonas vaginalis and the parent strain as well, kindly provided by Dr Linstead at Wellcome Laboratory, Beckenham, England.

(5) Pentatrichomonas hominis (from American Type Culture Collection).

B.2 Collection and Handling of Specimens

B.2.1 Isolation of Trichomonas vaginalis

Specimens of vaginal fluid were collected, using a method similar to that of Robertson

et al. (1969) from female patients attending James Pringe House (the sexually-transmitted diseases clinic of the Middlesex Hospital, London, WC1) for the first time. The procedure was as follows: Polyester sponge cubes (Campell Brushes Ltd, Bellingdon Road, Chesham, Bucks) were cut into pieces approximately 1 x 1 x 2.5 cm. Each piece of sponge was placed into a clean 5 ml bijou bottle and autoclaved at 120°C for 30 minutes; the caps were tightened after the bottles were cool. Using a diamond pen, each autoclaved bottle containing a piece of sponge was numbered, wiped with a dry soft tissue, and weighed to 10^{-4} g. The bijou bottles were sent to the clinic, then marked again with the patient's number and date using adhesive labels. Doctors at the clinic kindly assisted us by collecting vaginal fluid using the following procedure:

Sponge-holding forceps (Hartman's "crocodile" oral forceps, A. Young and Son, Edinburgh, Scotland) were used to withdraw a piece of the sponge from the bottle, insert it into the patient's vagina to soak up the fluid present and then return it to the bottle, which was then closed. The bottles were collected from the clinic twice a day and no specimens were kept for more than 4 hours before being examined. Bottles awaiting collection were kept at room temperature. In these conditions cultured Trichomonas vaginalis suspended in normal saline and absorbed in sponge remained

viable for at least 5 hours (Ackers et al., 1975).

At the London School of Hygiene and Tropical Medicine, the patient's number and serial number of the bottle were recorded and the bottle reweighed after removing the adhesive labels and cleaning with acetone. This enabled an estimate of the weight of the secretion collected to be made.

To recover the secretion, one millilitre of sterile normal saline was added to each sponge and the bottle was transferred to the top socket of a sterile Hemmings filter holder without filter pad (R.B. Turner, East Finchley, London, England), replacing the bijou bottle already there. It is important that the sponge bottle is screwed to the correct end of the filter, the end without the projecting steel rim, otherwise much secretion is lost. Externally this end is marked "H.A. Jones". The receiving bottle was marked with the patient's number and date and the whole assembly with sponge uppermost was spun in an MSE bench centrifuge at 900 G for 10 minutes. After centrifugation the receiver contained a clear supernatant and a pellet. The sponge bottle and Hemmings filter were removed and the lower bottle containing the spun secretion was temporarily capped. The clear supernatant was withdrawn with a one millilitre sterile disposable syringe, the volume was recorded, and then transferred to plastic polypots (Hughes & Hughes, Harold Wood, Essex). After labelling with the patient's

number and date the polypot was stored at -20°C.

Using a one millilitre syringe, 0.2 ml of culture medium without antibiotics (see below for details of medium) was withdrawn from a 15 ml bottle (previously warmed at 37°C for 4 hours) and mixed with the pellet in the receiving bottle. One drop was used to make a wet film, another drop was spread on a plate of SL agar (Rogosa et al., 1951a and b) for isolation of Lactobacillus acidophillus (see below for details of medium), and the remainder was used to inoculate 15 ml culture medium for T. vaginalis containing antibiotics in a 15 ml screw-capped bottle. The medium bottle was labelled with the patient's number and date and incubated at 37°C. Minimum air space in the bottle, and non-inhibitory white rubber wads are necessary to obtain growth from a small inoculum (Lumsden et al., 1966).

Wet films were examined under phase contrast at 400 x total magnification, and the following was noted:

(a) Trichomonas vaginalis = presence or absence

(b) Epithelial cells = few, moderate and many on arbitrary scales

(c) Pus cells = few, moderate, and many on arbitrary scales

(d) Blood cells = presence or absence.

Cultures were examined daily for colour change indicating

the production of acid, and microscopically for the presence of Trichomonas vaginalis. Cultures still negative for T. vaginalis after 14 days incubation were discarded.

B. 2.2 SL agar medium

Trypticase (BBL)	10 g
Yeast extract (Difco)	5 g
KH_2PO_4	6 g
Ammonium citrate [$(NH_4)_2HC_6H_5O_7$]	2 g
*Salt solution	5 ml
Glucose	20 g
Sorbitan monooleate (Tween 80)	1 g
Sodium acetate ($NaC_2H_3O_2.3H_2O$)	25 g
Glacial acetic acid (99.5%)	1.32 ml
Agar	15 g

Distilled water to one litre; pH 5.4

*Salt solution: $MgSO_4.7H_2O$ (11.5 g); $MnSO_4.2H_2O$ (2.86 g); $FeSO_4.7H_2O$ (0.68 g) and distilled water to 100 ml.

B.2.3 Culture medium for T. vaginalis

Organisms were grown in the medium of Lumsden et al. (1966), modified (Ackers et al., 1975) by the replacement of liver digest by Neutralized Liver Digest (Oxoid), and by altering the pH of the

buffer component to 6.8. The final pH of the medium was checked and adjusted to 6.8 if necessary using 1 N HCl. Originally the medium was derived from that described by Feinberg and Whittington (1957), with a view to improving the ease of manipulation of its characteristics for experimental purposes. The final constitution of the medium and methods of preparation are given below. Glass-distilled water and AnalaR reagents (British Drug Houses, Poole, Dorset) were used, except as otherwise stated. The medium contains Penicillin G (1,000 units/ml), and streptomycin sulphate (0.5 mg/ml); nystatin (250 µg/ml) was added to the medium only for the primary isolation of T. vaginalis from vaginal secretions.

B.2.4 Stock solutions

1. Neutralized Liver Digest (Oxoid): 40 g dissolved with gentle agitation to avoid frothing, in 1000 ml water, distributed into 3 x 300 ml sterile bottles and stored at -20°C.
2. D (+) Glucose (AnalaR): 27.74 g dissolved in 500 ml water, distributed in 5 x 100 ml sterile bottles and stored at -20°C.
3. Inactivated calf serum (Wellcome): 100 ml amounts, stored at 4°C.

4. Solution (A) (salts):
   - 9.01 g sodium chloride (NaCl) dissolved in 1000 ml water
   - 1.150 g potassium chloride (KCl) dissolved in 500 ml water
   - 0.978 g magnesium chloride ($MgCl_2.6H_2O$) dissolved in 100 ml water
   - 1.143 g calcium chloride ($CaCl_2$) dissolved in 100 ml water.

   The salt solutions were mixed in the ratio: 100 : 4 : 3 : 1, then dispensed in 100 ml sterile bottles and autoclaved.

5. Solution (B) (buffer):
   - 12.0 g sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) dissolved in 500 ml water
   - 36.9 g disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) dissolved in 100 ml water.

   Mixed in the ratio 400 : 600; distributed in 100 ml sterile bottles and autoclaved.

6. Bromocresol purple (Hopkins and Williams, Chadwell Heath, Essex):
   - 3 g suspended in 100 ml solution (A).

7. Sodium mercaptoacetate (British Drug Houses, Laboratory Reagent):
   - 1 g to be added to each litre of medium.

Glass-distilled water was used for the preparation of all solutions. Preparation of medium for one

litre:

add at room temperature:

- 392.5 ml solution (A)
- 300.0 ml neutralized liver digest solution
- 100.0 ml D(+) glucose solution
- 100.0 ml inactivated calf serum
- 100.0 ml solution (B)
- 1.0 ml bromocresol purple
- 1.0 g sodium mercaptoacetate.

Mix gently to avoid frothing and adjust pH to 6.8, then add antibiotics:

- Benzyl penicillin (B.P.) (Glaxo Laboratories, Greenford, Middlesex)
  1,000,000 units dissolved in 5 ml solution (A)
- 2.5 ml streptomycin sulphate (Glaxo Laboratories, Greenford, Middlesex)
  1 g (potency 745 units per mg) in 5 ml solution (A).

When all ingredients were mixed, sterilization was carried out using either a Seitz-filter or a membrane filter.

B.2.5 Seitz filter (HP/EKS)

The filter consists of an asbestos-cellulose pad with a follicular upper surface supported by a woven base. For sterilization, the stainless steel holder, filter pad and receiver were assembled with

the bolts loose, and then wrapped in aluminium foil and autoclaved for 30 minutes at 108°C (5 lb/sq in). The stainless steel funnel and the siphon were each wrapped separately and autoclaved in the same way. Suction was induced through the side arm in the receiver with a water pump. Before use, the pad was saturated with sterile saline and only then was the holder firmly screwed down, by gently tightening each screw in turn, so that the two halves of the holder gripped the pad evenly. According to Meynell and Meynell (1970), the drop in pressure across the pad should be kept as constant and as small as possible, and must not exceed 26 cm Hg (5 lb/sq in). Nystatin (250,000 units), which is particulate rather than soluble was added aseptically after filtration if required.

B.2.6 Membrane filtration

A slightly different procedure was employed when the medium was sterilized by membrane filtration. The medium prepared without serum and antibiotics, was clarified by passing under pressure through Whatman No. 3 filter paper. Prefiltration was through a series of non-sterile 293 mm diameter Millipore membranes (nitrocellulose membrane filters) - 1.2 µm, 0.8 µm, and then 0.45 µm pore size. The

filtered medium was kept at 4°C while the whole assembly of Millipore filter with 0.22 µm pore size membrane filter was sterilized. Penicillin and streptomycin were added and the medium was passed through 0.22 µm pore size membrane filter.

The medium was prepared in 5 litre amounts and after filtration dispensed aseptically in 450 ml lots, from the collection flask into sterile, 500 ml capacity, round, boro-silicate glass bottles. These bottles would withstand freezing. The filtered medium was stored at 4°C or -20°C. Before use 10% v/v of inactivated calf serum (Wellcome) was added together with 250,000 units/litre of Nystatin if required. Dispensing of medium in small bottles (either 5 ml bijou or 15 ml Lowenstein) was carried out in a sterile hood.

After sterilization, samples of the medium were left at room temperature (20°C to 26°C) for 48 hours, another group were incubated at 37°C for 48 hours, after which both examined for sterility. In case of contamination the whole batch was discarded. Bottles of medium were not kept at 4°C for more than two weeks, if longer storage was necessary the medium was frozen.

B.2.7 Stabilation of T. vaginalis

Protozoa, unlike bacteria and viruses,

cannot be easily preserved by lyophillization (Lumsden et al., 1973). Stabilates of T. vaginalis were therefore prepared by cryopreservation. By cryopreservation the biological characters are not fundamentally altered, and the properties of individual samples of a stabilate (given impeccable conditions of cryopreservation) remains consistent for periods of at least several years. On prolonged serial passage, the biological characters may be changed. The procedure described by Lumsden et al. (1966) for low temperature preservation of T. vaginalis and Lumsden et al. (1973) (for trypanosomes) were adopted using 8% (v/v) final concentration of dimethylsulphoxide as cryoprotectant.

The culture of organisms which it was desired to preserve was centrifuged at about 600 g for 10 minutes. The supernatant was removed with a sterile pipette and discarded, and the deposited organisms resuspended in about 0.5 ml of fresh medium previously warmed at 37°C for two hours. 0.2 ml of this suspension was mixed with 0.8 ml of 10% (v/v) dimethylsulphoxide in fresh medium as cryoprotectant. The mixture was then distributed with a sterile pasteur pipette to sterile lymph tubes (Plowden and Thompson, Stourbridge, England: 4 in long and with a nominal 1 mm outside diameter) held in a rack (described by Cunningham et al., 1963). The amount of suspension in each tube was about 20 µl. The lymph tubes were

then sealed at both ends in a microburner, removed from the rack and placed in a paper capillary tube container, previously numbered (both in tab and body) and coded. The container with the capillary tubes was inserted into the aluminium heat reservoir and transferred into insulating jackets. The insulating jacket was closed and sealed with a strip of adhesive tape and deposited in the solid CO_2 cabinet or in the mechanical deep freeze at $-70^{\circ}C$. When the temperature of the contents of the insulating jacket approximates to that of the freezer (after 4 hours but usually left, conveniently, overnight (Lumsden et al., 1973)), the tubes were ready for transfer to the permanent store in liquid nitrogen ($-196^{\circ}C$).

B.2.8 Preparation of clones for T. vaginalis

Without special precaution, T. vaginalis strains and stabilates may be genetically heterogenous; pure populations may be obtained by growing them up from single organisms. Such populations are termed "clones" (Magara et al., 1953; Asami et al., 1955; Samuels and Stouder, 1960: Samuels, 1962; Cavier et al., 1964; Hollander and Frost, 1965; Lumsden et al., 1973; Ivey, 1975; Hollander, 1976). Different methods have been tried for preparation of clones both in agar and in liquid medium. The

procedures adopted were as follows:

B.2.9 Micromanipulation method in liquid medium

A method similar to that of Roigas (1969) was adopted. Using modified Lumsden medium, 10-fold serial dilutions of a thoroughly mixed 24 - 48 hours old culture of T. vaginalis were made until dilution is reached such that single organisms could be picked up in a drop of suspending medium. A sterile fine tipped pasteur pipette was placed in a suspension from which clones were to be prepared, the fine-tipped pipette was allowed to take up a small volume of the suspension, and with a teat, about 2.0 µl of suspension was dropped on to a sterile coverslip (approximately 5 x 5 mm) placed on a clean slide with moist chamber. This was quickly examined under a low power microscope to confirm the presence of a healthy-looking, motile, single organism of T. vaginalis. The presence of only one organism was confirmed by at least one other observer. A coverslip carrying a single T. vaginalis was quickly transferred with sterile forceps into 5.0 ml bijou bottle containing the warmed culture medium. The cultures were divided into two groups, one group was incubated in anaerobic conditions (10% CO_2 in hydrogen) using McIntosh and Filde's jar, and the other group was incubated in air,

both groups at 37°C. The cultures were checked for the growth of T. vaginalis on days 5, 7, 9, 11, 14. Cultures which remained negative after day 14 were discarded and recorded as negative.

B.2.10 Micromanipulation method in agar medium

Using modified Lumsden medium, agar plates were prepared as follows: special agar (L28, Oxoid) to give a final concentration of 0.45% was dissolved in a buffer solution (B) by heat and cooled to 40°C. The other components of the medium, already mixed, were warmed to 40°C in a water bath, and then mixed with the buffer solution containing the agar. The complete medium was poured in petri-dishes (100 by 15 mm) and left to solidify. Using the micromanipulation method already described, and a 20 x 20 mm coverslip, the whole coverslip was placed, inverted on to the surface of the plate using sterile forceps. The petri dishes were then incubated inverted in a McIntosh and Filde's jar containing 10% carbon dioxide in hydrogen. The jars were opened on days 5, 7, 9, 11, 14 and the plates were examined.

B.2.11 Pour-plate methods

Method (A): Cultures of T. vaginalis were counted and diluted until 0.5 ml contained 10 - 100

organisms. This volume was then mixed with 36 ml modified Lumsden medium containing 0.4% final concentration of agar (L28, Oxoid) just before pouring into a petri dish. Incubation and checking of the plates were carried out as above.

Method (B): The method used was essentially similar to that of Hollander (1976) with slight modification. The medium used was the TYM medium of Diamond (1957) modified by substitution of ascorbic acid for cysteine (in accordance with the demonstrable superiority of ascorbic acid as a reducing agent in *T. vaginalis* media (Hollander and Frost, 1965)) and the addition of phosphate buffer (omitted from Diamond's published medium) and the use of potassium salts since excess sodium appears to be inhibitory. The ingredients of the medium, except serum were:

Trypticase peptone (BBL, code 11921)	20.0 g
yeast extract (BBL, code 11928)	10.0 g
maltose	5.0 g
ascorbic acid	1.0 g
KCl	1.0 g
$KHCO_3$	1.0 g
KH_2PO_4	1.0 g
K_2HPO_4	0.5 g
$FeSO_4$	0.1 g
agar (L28, Oxoid)	3.6 g
H_2O (glass-distilled)	900 ml

The ingredients of the medium were dissolved with heat, dispensed in 36 ml quantities into 100 ml bottles and autoclaved at 15 lb pressure for 15 minutes. Four ml of horse serum (uninactivated or inactivated, Wellcome) and 0.1 ml of fluid medium containing the inoculum of T. vaginalis to be cloned (diluted to contain 10 - 100 organisms) was then added to the bottle of melted medium, the contents mixed well, and the suspension poured into 100 by 15 mm petri dish. The plates were incubated right side up and care was taken not to tilt them since the medium was very soft. Incubation was in a McIntosh and Filde's jar containing 10% carbon dioxide in hydrogen at 37°C. The jar was opened on days 5, 6, 7 and the plates were examined.

B.2.12 Use of anaerobic jar

McIntosh and Filde's anaerobic jars used were obtained from Baird and Tatlock Ltd. The metal jar (8 x 5 inches) is provided with a lid that can be clamped down to make it airtight. The lid is furnished with two tubes and valves. A capsule containing pellets of palladiumized aluminia is suspended under the lid. This acts as a catalyst in the reaction of hydrogen with oxygen which leads to anaerobiasis. A side arm is connected to a small

glass tube half filled with diluted methylene blue solution; disappearance of the blue colour indicates satisfactory anerobiasis. When required for use, the jar was cleaned and dried. After putting the plates or culture bottles (with loose caps) in it, the lid was replaced and clamped on tight. The air inside was evacuated through the outlet valve by using a water pump. A gas mixture of 10% carbon dioxide in hydrogen (BOC) was admitted to the jar via a football bladder attached to the gas cylinder. The jar was then incubated at 37°C. Before opening the jar it is necessary to release the pressure by opening both valves away from any source of flame.

B.2.13 Examination of plates for clones of T. vaginalis

To avoid excessive moisture, it is advisable not to incubate more than 4 plates in one jar. All the plates to be examined on one day were incubated in the same jar. To avoid contamination the plates were examined unopened, using the naked eye and a very low power objective, for the presence of colonies of T. vaginalis. If colonies were found, one colony was picked up with a bacterial loop, suspended in a drop of sterile saline on a clean slide and examined microscopically for the presence of active organisms. Several colonies were examined to confirm that they

consisted of T. vaginalis and not bacteria or fungi; they were then transferred to medium to be grown up as clones.

B.2.14 Efficiency of plating for T. vaginalis

The efficiency of plating was checked by the following method: the viability of the cultures to be cloned was established by using trypan-blue exclusion (see below); then 10-fold serial dilutions were made and different inoculum was used in plating as above. The number of colonies was compared with the size of the inoculum used.

B.3 Isolation of intestinal Trichomonads from monkeys

The stool specimens from monkeys at the London Zoo were kindly supplied by Mr Peter Sargeaunt and Mr John Williams at London School of Hygiene and Tropical Medicine

B.3.1 Isolation of intestinal Trichomonads from a monkey in modified Lumsden medium

The stool specimen from the monkey was suspended in sterile saline pH 7.2, and inoculated into three groups of 5 ml bijou bottles containing

modified Lumsden medium. The first group of culture bottles contained modified Lumsden medium alone; the second group of culture bottles contained the modified Lumsden medium as an overlay on saline-agar slopes (see below). The third group of culture bottles were exactly the same as the second group except that rice starch powder (Oxoid) was added. Each group of culture bottles was divided further into two groups, one of them was incubated anaerobically (using McIntosh and Filde's jar) and the other was incubated in air. Both were left at 37°C. None of these bottles was found to be positive for live Trichomonads. We therefore tried using Robinson's (1968a) medium for isolation of the intestinal Trichomonads from monkeys in the same way as it is used for the laboratory diagnosis of human parasitic amoebae.

B.3.2 Isolation of intestinal Trichomonads from a monkey in Robinson's medium

The stool specimen suspended in sterile saline was incubated in bijou bottle containing Robinson's medium (for details of Robinson's medium, see below): 2 ml of 0.05M phthalate solution and 1 ml equal volume of medium "R" containing Es.coli strain B (NCTC No. 10537) and uninactivated horse serum (Wellcome Laboratories, No. 3) as a liquid overlay on

the saline agar slope. At the time of inoculation 0.5% (w/v) erythromycin solution, 10 mg rice starch (judged on blade or bacterial loop) and two drops of bactopeptone were added. The culture bottles were divided into two groups, one group was incubated anaerobically using McIntosh and Filde's jar at 37°C, and the other group was incubated as usual in the incubator at 37°C. The culture bottles were examined every day and the supernatant, carefully without disturbing, was subcultured in the same medium.

B.3.3 Axenic culture of intestinal Trichomonad from monkey

The monkey intestinal Trichomonad was freed from amoebae and bacteria by the addition of antibiotics and by omitting Es. coli from the medium. Under these conditions the bacteria necessary for growth of the amoebae are inhibited and the latter slowly die out.

B.3.4 Robinson's medium (Robinson, 1968a)

This is a diphasic medium and consists of a saline-agar slope and a liquid overlay.

Saline-agar slopes: 15.0 g fine agar powder (BDH) and 7.0 g sodium chloride were dissolved by heating in a litre of glass distilled water. The

solution was distributed in 2.5 ml quantities in quarter-ounce screw-capped glass bottles (bijou) or in 10 ml quantities in universal bottles. The bijou bottles had rubber-lined perforated screw caps to facilitate easy inoculation and minimize the risk of contamination. The bottles were sloped and allowed to set after autoclaving (15 lbs pressure for 15 minutes). They were stored at room temperature.

The overlay: was made up from the following solutions:

Antibiotics: 0.5 g erythromycin powder (Abbot Laboratory) was dissolved in 2.5 ml 70% ethanol in a sterile container and left in the refrigerator at 4°C for 2 hours. 97.5 ml sterile glass-distilled water was added aseptically. The solution was distributed in 4.0 ml volumes in bijou bottles and stored at 4°C.

Bactopeptone: 20.0 g bactopeptone (Difco) were distributed in 100 ml sterile glass-distilled water. The solution was dispensed in 4 ml volumes in bijou bottles, sterilized by autoclaving (15 lbs pressure for 15 minutes) and stored at 4°C. 10% Lablemco (Oxoid) was substituted for bactopeptone when stock cultures were failing.

Starch: Rice starch powder (Oxoid) was distributed in small amounts in bijou bottles, sterilized by autoclaving (15 lbs pressure for 15 minutes) and stored at room temperature.

Phthalate solution

204.0 g potassium hydrogen phthalate were dissolved in 100 ml freshly prepared 40% sodium hydroxide solution. This was made up to 2.0 litres with glass-distilled water; the pH was adjusted to 6.5. The solution was dispensed in 100 ml medical flat bottles, autoclaved (15 lbs pressure for 15 minutes) and stored at room temperature. When required for use, the buffer stock solution was diluted 1:10 with sterile glass-distilled water to make a 0.05 M solution.

Medium "R"

The following ingredients were dissolved in one and a quarter litre of glass-distilled water:

62.5 g sodium chloride
25.0 g citric acid monohydrate
6.25 g potassium dihydrogen orthophosphate (anhydrous)
12.5 g ammonium sulphate
0.625 g magnesium sulphate heptahydrate
50 ml lactic acid (BDH, 90.08%).

This stock solution, pH 7.0, was stored for at least 4 weeks before use to avoid change of pH on autoclaving (Robinson, 1968a). For use, 7.5 ml freshly prepared

40% sodium hydroxide and 2.5 ml 0.04% bromothymol blue were added to 100 ml stock solution and made up to one litre with glass-distilled water. The medium was dispensed in 20 ml quantities in 100 ml medical flat bottles, autoclaved (15 lbs pressure for 15 minutes) and stored at room temperature.

Basal medium "BR"

A 10.0 µl Escherichia coli B stabilate, was obtained from National Collection of Type Cultures (No. 10537), was inoculated into 4 ml peptone water in a bijou bottle, incubated at 37°C for 24 hours and then left at room temperature overnight. 0.1 ml of this was added to 20 ml medium "R" in medical flat bottles and incubated at 37°C for 24 hours. The BR was stored at room temperature for 2 days before it was used to allow Es. coli to grow.

Basal medium "BRS"

400 ml uninactivated horse serum (Wellcome Laboratories, No. 3) was removed from -20°C storage and allowed to thaw on the bench. It was aseptically distributed in 20 ml volumes in universal bottles. The bottles were kept at 4°C. To make BRS, 20 ml serum was added to 20 ml BR and incubated at 37°C for 24 hours. It was subsequently stored at room temperature.

B.4. Electron Microscopy of the Monkey Trichomonad

B.4.1 Harvesting and fixation

Organisms from 2-day old cultures were sedimented at 400 x g for 5 minutes. The supernatant were discarded and 3% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 containing 0.1% $CaCl_2$ added to the pellet which was then kept at $4^{o}C$ overnight.

B.4.2 Washing and dehydration

Materials were washed in 0.1M cacodylate/0.2M sucrose buffer, pH 7.4, four times during twelve hours, and post-fixed for 2 hours in 1% osmium tetroxide in the cacodylate buffer. Further washing was done with the cacodylate/sucrose buffer followed by distilled water for two hours and dehydration achieved using graded methanols, with the material remaining for 30 minutes in 3% uranyl acetate in 30% methanol.

B.4.3 Embedding and cutting

The specimens were embedded in Fluka araldite via propylene oxide and cut on a Huxley Cambridge Automatic Ultramicrotome.

B.4.4 Examination

Specimens were lead-stained and examined with an AEI EM 801 electron microscope using an accelerating voltage of 80 KV and photographed on Ilford SP 353 film.

B.5. Preparation for Isoenzyme Electrophoresis

B.5.1 Organisms

(A) Freshly isolated from man:
Isolations were made from patients attending James Pringle House (the sexually-transmitted-disease clinic of the Middlesex Hospital, London, W1) for the first time.

(B) From other sources:

1. Three different isolates from faeces of monkeys at the London Zoo naturally infected with an intestinal trichomonad, were kindly provided by P.G. Sargeaunt and J. Williams of London School of Hygiene and Tropical Medicine.
2. Two isolates of T. vaginalis from Italy, said to be infective for mice and kindly provided by Professor Cappuccinelli.
3. Two isolates from hamsters infected by one isolate of human origin; passaged intravaginally for a

period of approximately two years. These isolates were referred to as Beckenham strain and were kindly provided by Dr Linstead of Wellcome Research Laboratory, Beckenham.

4. Pentatrichomonas hominis from American Type Culture Collection (ATCC 30 000).

Summary of T. vaginalis strains is given in Table 26.

B.5.2. Cultivation

All organisms were cultivated in modified Lumsden's medium for T. vaginalis unless otherwise indicated. Two capillary lymph tubes containing the cryopreserved organisms were placed in cold water to thaw. Using a piece of sterilised cotton or gauze moistened with absolute alcohol, the capillary tubes were wiped and left for two minutes. Each tube was cut at both ends by diamond pen and by using "holey-blower" teat (Figure 7) the contents were inoculated into 5 or 15 ml bottles containing modified Lumsden's medium; the bottles then were incubated at 37°C.

For lysate preparation, large amounts of organisms were grown in approximately 500 ml of modified Lumsden's medium in 500 ml medical flat bottles using cultures in 5 or 15 ml bottles in exponential

growth phase as inoculum. Antibiotics, 1000 IU/ml of penicillin and 500 µg/ml of streptomycin sulphate were added to the culture medium and the cultures were incubated horizontally at 37°C.

Because some isolates had originally been received in media other than modified Lumsden's medium we prepared lysates of organisms cultured in different media as well as in the modified Lumsden's medium. For instance, the Beckenham hamster strains and its parent T. vaginalis strain were cultured in Stenton's medium (Stenton, 1957; see below for details of medium) as well as in modified Lumsden's medium; other isolates of T. vaginalis were cultured in Hollander's medium (Hollander, 1976) without agar. This was done to look for any effect on the isoenzyme patterns of growth in different media; it was not possible to grow the intestinal trichomonads isolated from monkeys in modified Lumsden's medium and therefore we used lysates prepared from cultures in Robinson's medium.

B.5.3 Stenton's medium

Ascorbic acid	1.0 g
L-cysteine hydrochloride	0.6 g
Oxoid dehydrated liver infusion	0.25 g
Glucose	2.5 g
Hartley's digest broth	460 ml

Added at time of use:

Horse serum	8 per cent
Penicillin	500 units/ml
Streptomycin	500 units/ml

pH adjusted to 6.0; sterilization and storage were the same as mentioned before.

B.6 Preparation of Trichomonad Extracts

B.6.1 Harvesting of organisms

Organisms of T. vaginalis, the monkey trichomonads and the Beckenham strain were harvested in late exponential growth phase by centrifugation in 250 ml clean plastic bottles in a MSE high speed "18" refrigerated centrifuge at 1000 g for 25 minutes at 4°C. The supernatant was discarded using a 20 ml plastic disposable syringe, the pellet was resuspended in about 10 ml phosphate buffered saline pH 7.2 and then transferred into 10 ml polypropylene tube and centrifuged at 1000 g for 25 minutes at 4°C. This process of washing was repeated three times, unless the pellet was very small, when it was only washed twice.

B.6.2 Lysing

After harvesting and washing of the organisms,

the pellet was mixed with an equal volume of enzyme stabiliser solution (Kilgour and Godfrey, 1973) containing:

- 2.0 mM ethylenediaminotetraacetic acid (EDTA), to protect the enzymes of interest by inhibition of proteases (O'Sullivan and Morrison, 1963).
- 2.0 mM dithiothreitol (DTT), the function of which is to protect thiol compounds such as sulfhydryl (-SH) groups in proteins against oxidation (Cleland, 1964; Hopkins, 1975).
- 2.0 mM ε-aminocaproic acid (E-ACA), to prevent the hydrolysis of certain peptide chains by competitive inhibition of peptidases at the active site (Webb, 1966).

The suspension was mixed with a vortex mixer and then kept on ice for approximately ten minutes to start the process of lysis. The organisms were then completely lysed by freezing in a -20°C deep freeze for 24 hours (Kilgour and Godfrey, 1973; Kilgour et al., 1974; Godfrey and Kilgour, 1976).

B.7 Preparation of Lysate Beads

B.7.1 Thawing and centrifugation

The lysed organisms were thawed in ice and then centrifuged (initially at 100,000 x g, later

at 34,000 x g) at 4°C for 45 minutes, to remove as much as possible of the particulate matter. The clear supernatant (crude extract) was removed and placed on ice; as rapidly as possible it was frozen in beads.

B.7.2 Beading

Using a fine-tipped pasteur pipette or capillary tubes (1 mm outside diameter, 10 cm long) with "holey-blower" teat, the supernatant was released as small droplets into a clean plastic container containing liquid nitrogen to form beads of 10 - 40 µl. Beads of each strain were transferred into liquid nitrogen-cooled small vials (approximately 1 ml) closed with a screw cap which had previously had a minute hole made in it to allow nitrogen to enter and equalize the pressure when the vials were finally sorted in liquid nitrogen. The vial was appropriately labelled using strips of lassobands (Lasso Ltd), showing the reference number of the strain and date of storage.

B.7.3 Storage

Aluminium canes were used to hold the small vials containing the beads. Each cane contains 6

spaces enabling 6 small vials to be held vertically. Each cane was numbered from the top margin , replaced in numbered canisters and stored in liquid nitrogen until required. It is essential that the process of beading and storage should be done without delay, otherwise much of the activity of certain enzymes is lost.

When the extracts were needed for analysis or electrophoresis, one or more beads were taken, placed into the wells (U-shaped) of a microtitre plate, numbered, thawed and kept on ice for immediate use.

To standardise the work on isoenzymes, it was necessary to carry out protein estimation and assay of enzyme activities for samples of Trichomonas extract before using thin layer starch-gel electrophoresis.

B.8 Protein Estimation

The protein concentration of some extracts of Trichomonas was determined spectrophotometrically by the Folin phenol method of Lowry et al. (1951). The method is slightly modified. Bovine serum albumin was used as standard.

B.8.1 Reagents

Solution (A) (stock): 2% (w/v) sodium carbonate (Na_2CO_3) in 0.1M NaOH.

Solution (B) (stock): 0.5% copper sulphate ($CuSO_4 . 5H_2O$) in 1% sodium potassium tartrate

Solution (C): freshly prepared, by mixing 49 ml of solution (A) with 1 ml of solution (B)

Solution (D): freshly prepared ≈ 1N diluted Folin reagent: 10 volumes of Folin-Ciocalteau BDH reagent plus 10 volumes of glass-distilled water

Protein standard solution: bovine serum albumin solution (supplied by Sigma as 5%) of known concentration (1 mg/ml).

B.8.2 Procedure

In successive tubes (except the blank tube) 10 µl, 25 µl, 50 µl, 75 µl and 100 µl of the bovine serum albumin solution (1 µg/µl) were dispensed, followed by enough glass-distilled water to give a final volume of 100 µl in each tube. Final concentrations of protein were 100, 250, 500, 750 and 1000 µg/ml respectively. 100 µl glass-distilled water was added to the blank tube. 100 µl of normal sodium hydroxide solution was added to each tube including the blank. One millilitre of solution (C)

was added to all tubes, the contents of each tube mixed well with a vortex mixer and left for 10 minutes at room temperature.

One hundred microlitres solution (D) was then added to all the tubes and the contents of each tube again mixed well before being left for 30 minutes at room temperature. After this time the blue colour was fully developed; all optical densities were read within 3 hours of this point.

B.8.3 Reading

The contents of each tube were transferred to clean glass cuvettes (Silica microcell, 1 cm light path) and placed in the spectrophotometer (Pye Unicam-SP 1700 ultraviolet). The absorbance of the sample was measured at 660 nm wavelength against a reagent blank. A typical standard curve is shown in Figures 1,2,3.For estimation of protein content of Trichomonas extracts the procedure was as follows:

Samples to be estimated were diluted ten-fold with glass-distilled water. One hundred microlitres of the diluted sample was analysed by the same procedure as that used for the preparation of the standard curve. The absorbance at 660 nm wavelength was read against a reagent blank. The absorbance reading was recorded and then used to calculate the protein concentrations of the Trichomonas

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extracts from the standard curve. All the estimations were carried out in duplicate.

Since it was not possible to assay all the samples at the same time, the full range of standards were included every time. Results of protein estimations are shown in Tables 14, 15, 16, 17, 18, 19.

B.9 Assay of Enzyme Activities

Some lysates were assayed spectrophotometrically to determine the total activity of the enzyme of interest. All assays were carried out in a Pye Unicam-SP 1700 Ultraviolet recording spectrophotometer, fitted with a Grant circulator (to control the temperature of the cuvettes) set at 30°C during the experiments. Stock solutions of enzyme substrates were mixed with a small quantity of diluted extract (using Gilson Pipetman adjustable automatic pipettes) in silica microcells (cuvettes) of 1 cm light path.

Samples with high enzyme activity were diluted with 0.2M phosphate buffer pH 7.4 (Dr V. Kilgour, personal communication) to a level which could be conveniently measured and gave linear reaction with respect to time. Less potent extracts were used undiluted. Results of these enzyme assays are shown in Table 12.

The reagent and procedures used for measuring the activity of the enzymes are as follows:

(1) LACTATE DEHYDROGENASE (LDH) : (E.C.1.1.1.27)

$$\text{Lactate} + \text{NAD} \overset{\text{LDH}}{\rightleftharpoons} \text{Pyruvate} + \text{NADH}_2$$

The activity of the enzyme was assayed by the method of Lanham and Scott (personal communication). The reaction is based on the reduction of lactate to pyruvate and the activity was determined by measuring the rate of reduction of NAD to $NADH_2$ (increase in the extinction at 340 nm wavelength), in the presence of lactate dehydrogenase.

(a) Reagents

The reaction mixture contained:

Stock solution: LDH (lactate as a substrate)

Tetra sodium-pyrophosphate ($Na_4P_2O_7$, BDH)	1.24 g
DL-lactic acid	0.4 ml
NAD^+ (Sigma)	0.22 g

The above mixture was dissolved in about 30 ml glass-distilled water, adjusted to pH 8.8 with 0.1M NaOH, and made up to 56 ml.

(b) Procedure

0.7 ml of the above stock solution together with 20 µl distilled water (to standardize volume) were pipetted into the cuvette and a known amount of the sample to be assayed added. The cuvette was covered with Parafilm and the contents mixed by gentle shaking. The cuvette was then cleaned with soft tissue and should be without any turbidity or fingerprints. The cuvette was placed immediately into the spectrophotometer. The reaction was carried out at 30°C. The rate of change in extinction at 340 nm wavelength was recorded and the enzyme activity was calculated (see below). The blank used was either a clean empty cuvette (i.e. air blank) or preferably a cuvette containing the sample and the reaction mixture without substrate.

(2) HEXOKINASE (HK) : (E.C.2.7.1.1)

The spectrophotometric assay of HK is based upon the following reactions:

$$\text{D-Hexose} + \text{ATP} \xrightleftharpoons{\text{(HK)}} \text{D-Hexose-6-phosphate} + \text{ADP}$$

$$\text{D-Hexose-6-phosphate} + \text{NADP} \xrightleftharpoons{\text{Glucose-6-phosphate dehydrogenase}} \text{6-phospho-gluconate} + \text{NADPH}_2$$

The activity of the enzyme was assayed by the method of Lanham (personal communication). This assay is based on the measurement of the reduction of NADP to NADPH (increase in the extinction at 340 nm wavelength) accompanying the conversion of D-Hexose-6-phosphate to 6-phosphogluconate.

(a) Reagents

The reaction mixture (freshly prepared) contained:

Tris/HCl buffer pH 8.0 (0.3M)	1.0 ml
$MgCl_2$ (0.1M)	0.3 ml
D-glucose (10.0 mM)	0.3 ml
ATP in Tris buffer (10.0 mM)	0.3 ml
NAD (10 mg/ml)	0.1 ml
Glucose-6-phosphate Dehydrogenase (G-6-PH) (Sigma, Type XV) (10 IU/ml)	0.1 ml
Water (glass-distilled	1.0 ml

(b) Procedure

Seven-tenths of a millilitre of the above reaction mixture was pipetted into the cuvette and a known volume of the sample to be assayed was added and mixed well as before. The cuvette was placed immediately into the spectrophotometer. The

reaction was carried out at 30°C. The rate of change in light absorption at 340 nm wavelength was recorded and the enzyme activity was calculated (see below). Blank used was either an empty cuvette or preferably a cuvette containing the sample and the reaction mixture without substrate.

(3) MALATE DEHYDROGENASE (MDH) : (E.C.1.1.1.37)

The activity of MDH was measured spectrophotometrically by making use of the following reaction:

$$\text{L-malate} + \text{NADH} \underset{}{\overset{\text{MDH}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{NADH}_2$$

The activity of the enzyme was assayed by the method of Lanham (personal communcation). The rate of the reaction was measured by following oxidation of malate to oxaloacetate, and was determined by measuring the reduction of NAD to $NADH_2$, determined by the absorbance at 340 nm wavelength.

(a) Reagents

Tris/HCl buffer (0.3M pH 7.4)	0.5 ml
$MgCl_2$ (0.1M)	0.15 ml
NAD (Sigma) (10 mg/ml)	0.20 ml
Water (glass-distilled)	2.10 ml

The above reaction mixture was freshly prepared.

(b) Procedure

0.6 ml of the reaction mixture and 0.15 ml of 0.02M sodium DL-malate was pipetted into the cuvette and a known volume of the sample to be assayed was added and mixed well. The cuvette was placed immediately into the spectrophotometer. The reaction was carried out at 30°C. The rate of change in light absorption at 340 nm wavelength was recorded and the enzyme activity was calculated (see below). The reference cell in this case contained air.

B.9.1 Calculation of Enzyme Activities

In general the reactions are started by addition of either the substrate and/or the extract sample to the solution of the reaction mixture contained in the cuvette. The rate of change in optical density (change of extinction) at 340 nm wavelength during the conversion of NAD to $NADH_2$ (MDH and LDH) or NADP to $NADPH_2$ (HK) was recorded over a period of 5 minutes at 30°C. The enzyme activities, which in all cases are directly related to the interconversion of oxidised or reduced forms of pyridine nucleotides, are measured in IU/ml (International Union of Biochemistry recommendation, Enzyme Nomenclature, 1973). The enzyme activity is calculated as follows:

$$\frac{\text{Change in O.D. per minute x total volume in cuvette x dilution}}{6.22 \text{ x volume of sample added}}$$

where 6.22 = the molar extinction coefficient of NADH.

The value obtained is equal to the number of micromoles of product formed per minute per millilitre of extract (crude enzyme) in an assay reaction mixture.

$$\text{Specific activity} = \frac{\text{enzyme activity (u/ml)}}{\text{concentration of protein in the sample solution (mg/ml)}}$$

B.10 Preparation of Thin Layer Starch Gel Electrophoresis

The thin layer starch gel method of electrophoresis used was adapted from those of Smithies (1955), Wraxall and Culliford (1968) and Kilgour and Godfrey (1973). The procedure for the preparation of the starch gel involves the following steps:

B.10.1 Dissolving the starch

Each gel plate (21.5 x 13.6 x 0.1 cm) requires about 40 ml of starch solution (see Figure 5). Dry hydrolysed starch (Sigma) was weighed out 25%

above the makers' recommendation for human serum electrophoresis which is given on the container. The starch was then added gradually to half the required volume of the gel buffer in a long-necked, round bottomed flask which could hold about twice the final volume. The remainder of the buffer was added (washing down any starch adhering to the neck of the flask) with continuous swirling to avoid lump formation. The starch was dissolved with constant swirling while being gently heated. Near its boiling point, the gel becomes less viscous, with small bubbles forming throughout the solution. At this point the flask was removed from the heat and degassed under vacuum. When the pressure was fully reduced, both large and small bubbles were seen coming from the starch solution (the vacuum was released when necessary to prevent boiling over). When the starch solution had been degassed it became clearer and bubbles stopped forming. The whole process of degassing was performed quickly to avoid the starch solution cooling and solidifying.

B.10.2 Pouring plates

Clear glass plates (21.5 x 13.6 x 0.1 cm) were placed on paper sheets. About 40 ml of hot solution of starch gel was poured in a continuous stream against the bottom edge strip of an electrophoretic plate until it extended approximately to

one third of the length of the plate. Using a perspex spreader (see Figure 4), the gel liquid was pushed from the lower to the upper edge of the plate with firm easy movement. The spreader was taken beyond the top of the plate, and then pulled back slightly to prevent runback of excess gel. The plate was left for about 10 minutes without disturbance to allow the gel to set before the underlying paper-sheet was pulled away with the excess of starch gel and then discarded. Plates were allowed to cool and then covered and transferred to a cold surface to become firm, preferably in moist environment (e.g. on the cooling plate in the electrophoretic tank already prepared with the suitable buffer).

B.11 Preparation of the Electrophoresis Tanks

B.11.1 Tanks

Tanks with close-fitted covers were made of perspex. Each tank has 4 compartments. The inner two comparments contain the electrodes and are connected to the outer compartments through holes. The suitable tank buffer was poured into each compartment and the buffer level was equalised in all compartments by gently tipping the buffer to one end

one third of the length of the plate. Using a perspex spreader (see Figure 4), the gel liquid was pushed from the lower to the upper edge of the plate with firm easy movement. The spreader was taken beyond the top of the plate, and then pulled back slightly to prevent runback of excess gel. The plate was left for about 10 minutes without disturbance to allow the gel to set before the underlying paper-sheet was pulled away with the excess of starch gel and then discarded. Plates were allowed to cool and then covered and transferred to a cold surface to become firm, preferably in moist environment (e.g. on the cooling plate in the electrophoretic tank already prepared with the suitable buffer).

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of the tank. Each tank required approximately one litre of tank buffer. (Fig. 6).

B.11.2 Cooling system

Excessive heat production will denature, inactivate or distort the banding pattern of the enzymes. It is essential to keep the gel plates as cold as possible during an electrophoresis run. The cooling system used (Grant, Cambridge, flow circulator and cooler FC15 and FC1) consisted of aluminium cooling plates with coiled tubes which were connected to a cooling pump and refrigerator unit. The aluminium plates were raised above the buffer in the electrophoretic tank. The system was checked for air bubbles, which interfere with the efficiency of cooling, then switched on and set at $0^{o}C$ at least one hour before electrophoresis run was started.

B.11.3 Wicks

Two current-carrying wicks for each tank were used, one on the anodal side and the other on the cathodal side of each gel plate. The wicks were made from cellulose sponge (Spontex) cut to match the width of the plates.

B.11.4 Power pack

The power source used was either a Chandos E32, capable of producing 250 mA at 400 V or a Heath-kit regulated high voltage Power Supply IP17, variable from 0-400 V and capable of continuous delivery of 125 mA at 400 V.

B.12 Preparation and Application of the Sample

Beads of extract sample (lysates) were taken out of liquid nitrogen store and placed in labelled wells in microtitre plate. The samples were thawed and kept covered on ice all the time during the experiment. If dilution was necessary 5% bovine serum albumin in water was used.

For each enzyme, a gel plate was placed onto a sheet of graph paper which was used as a guide. Slots were cut by means of a comb template, at 13 cm or 15 cm from the anode or midway between anode and cathode according to the conditions of each enzyme tested.

Two designs of slot comb templates are shown in Figure 4 one with 10 narrow teeth and the other with 8 wider teeth. Eight, ten or even twelve samples may be applied on one plate. Cotton threads were used as enzyme extract carriers. These threads were prepared by boiling white sewing cotton

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Two designs of slot comb templates are shown in Figure 4 one with 10 narrow teeth and the other with 8 wider teeth. Eight, ten or even twelve samples may be applied on one plate. Cotton threads were used as enzyme extract carriers. These threads were prepared by boiling white sewing cotton

in "Haemosol" for 15 minutes and rinsing 3 times in tap water followed by distilled water 3 times (Kilgour, 1976). The threads were cut shorter than the slots. The threads were saturated by capillary action in the prepared lysates (extracts), inserted into slots and tucked under the surface of the gel with fine-tipped forceps. The forceps were rinsed in distilled water and wiped with clean soft tissues between each application of different samples to avoid contamination. Different thicknesses of cotton thread were used according to the enzyme activity of each sample. Two threads, each carrying a different lysate in one slot enabled a more direct comparison of enzyme bands.

B.12.1 Transfer of the Gel Plate for Electrophoresis

The gel plate containing the samples was laid on a sheet of polyethylene terephthalate (Melinex, ICI, type 'O', gauge 23 mm) on the aluminium cooling plate. The cooling plate was kept at approximately 8°C. The gel plate was then covered by a clean thin glass plate (21.5 cm long x 13.6 cm wide x 3 mm deep) which was laid centrally widthwise across the gel parallel with the sample origin (slots). The wicks of "Spontex" were laid across

the exposed part of the gel closely abutting the central plate so that the wick dipped into the buffer in the exterior compartments of the tank. A clean thick glass plate (21.5 cm long x 13.5 cm wide x 5 mm deep) covered the whole to keep the wicks in position, to prevent drying out and to regulate both pressure on the gel and the flow of the buffer through the wicks. The tank was then covered by its lid and the power supply was turned on and the appropriate voltage selected. As many as six tanks could be wired in series depending on the capacity of the stabilised power supply. The factors affecting the electrophoresis and duration of run are the buffer pH and ionic strength, the current flow and the molecular size of the enzyme. After preliminary experiments, the duration was chosen so the bands were drawn sufficiently far from the origin line to give optimum resolution.

B.13 Staining the Gel

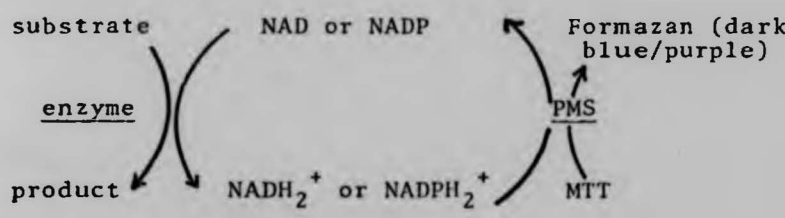
Using a dye staining method the sites of enzyme activity could be detected. Tetrazolium staining technique was used throughout our experiments. This method was used to detect PGI, PGM, HK, G6PD, MDH, LDH and aldolase activities on the gel. A perspex gel mould was placed on the gel in such a

way that the origin slots came into the middle of the mould. The specific staining reaction mixture for each enzyme (see below) was prepared approximately 10 minutes before the end of the run. In most cases this reaction mixture was made up of the following:

- buffer solution
- coenzyme (NAD^+ or $NADP^+$): in its absence the enzyme will not function.
- activator: some metal ions such as Mg^{++} and Mn^{++} are essential for the enzyme to catalyse the reaction.
- linking enzyme: in case of coupled reaction system.
- dye: methyl thiolzolyl tetrazolium (MTT) and Phenazine methosulphate (Sigma).
- hot agar (L28, 1.2%, Oxoid): approximately at 50°C and not allowed to set except on the gel plate.

The staining dye was the last to be added before the agar. The reaction mixture was immediately poured in the enclosed area of the gel and covered by a thin glass plate. The gel plate was kept in place without disturbance for about 3 minutes to allow the overlay to set, then incubated in the dark at 37°C. The gel plate with the overlay was examined over a bright light source every 5 to 10 minutes for

the development of dark blue/purple bands. The reduced MTT tetrazolium accompanying the reaction gives an insoluble formazan which was seen as dark blue/purple bands at the site of enzyme activity. The reaction is shown as follows:



Control plates were run for each enzyme for every extract and were electrophoresed under the same conditions as the experimental plates except that the specific enzyme substrate was omitted when the enzyme locating developer was being prepared.

B.14 Photography

The bands developed gradually and most of the plates were examined over a period of about two hours or even more, until the bands became diffuse. The electrophoretic enzyme patterns were photographed by placing the gel plate over a bright light fixed in a box. An exposure of 1/125 second and a lens of aperture f3.2 were used with a Polaroid camera and Polaroid Type 107C film. Copies were made from the same Polaroid pictures following the same procedure.

The enzymes studied by thin-layer starch gel electrophoresis and the enzymes studied by spectrophotometry are shown in Table 8 and Table 9, 12 respectively.

The conditions for electrophoresis and formazan development in agar overlay for the enzymes E.C.1.1.1.49. glucose-6-phosphate dehydrogenase (G6PD), E.C.1.1.1.40. malate dehydrogenase (decarboxylating) ($NADP^+$) (ME = "malic enzyme"), E.C.5.3.1.9. glucophosphate isomerase (GPI) and E.C.2.7.5.1. phosphoglucomutase (PGM), were based essentially on published methods and recommendations (Fildes and Parr, 1973; Carter and Parr, 1967; Bagester and Parr, 1973; Lanaham, personal communication; Kilgour, personal communication; Godfrey, personal communication). The conditions for the enzymes E.C.2.7.1.1. hexokinase (HK), E.C.4.1.2.13. aldolase (ALD), and E.C.1.1.1.27. lactate dehydrogenase (LDH) were adapted from those described by Harris and Hopkins (1976) for mammalian blood cells (Lanaham, personal communication; Miles, personal communication).

All the conditions were adapted with some modification to improve the resolution for Trichomonas vaginalis.

B.15 Buffer Solutions for Electrophoresis

All the buffers were prepared at room temperature (20°C - 22°C) and stored at 4°C for a maximum of one week. The preparation of solutions were as follows:

B.15.1 Tank Buffers

(1) 0.1M Tris/Maleic acid - 0.01M EDTA/$MgCl_2$ buffer pH 7.4 (Tris/maleate buffer)

Tris (hydroxymethyl aminomethane)	12.114	g
Maleic acid (ethane 1,2,dicarboxylic acid)	11.61	g
Ethylenediaminetetraacetic acid (EDTA)	3.72	g
Magnesium chloride ($MgCl_2.6H_2O$)	2.003	g
H_2O (distilled) to	1000	ml

Dissolve Tris, maleic acid and EDTA in 800 ml of distilled water. Neutralize the solution and adjust pH to 7.4 with 10N NaOH. Then add $MgCl_2$ and make up to one litre with distilled water. The final pH correction was with 10N NaOH solution. To avoid corrosion of electrodes (due to chloride ions) plug the inner chamber of tanks with a piece of cotton and use phosphate buffer in inner compartments (electrode chambers).

(2) 0.2M Na-phosphate buffer pH 7.0

Di-sodium hydrogen orthophosphate (anhydrous) ($NaHPO_4$)	17.4 g

Sodium dihyrodgen orthophosphate
($NaH_2PO_4.2H_2O$) 12.15 g
Water (distilled) to 1000 ml

(3) 0.1M Na-phosphate buffer pH 7.0
Disodium hydrogen orthophosphate
(anhydrous) ($NaHPO_4$) 8.7 g
Sodium dihyrogen orthophosphate
($NaH_2PO_4.2H_2O$) 6.1 g
Water (distilled) to 1000 ml

(4) 0.9M Tris/0.2M EDTA/0.02M Boric Acid/0.025M $MgCl_2$ Buffer pH 8.6 (Tris/Borate)
Tris(hydroxymethyl aminomethane) 54.503 g
Boric acid (H_3BO_3) 6.183 g
EDTA (ethylenediaminotetraacetic acid) 3.723 g
Magnesium chloride ($MgCl_2.6H_2O$) 2.541 g
H_2O (distilled) to 1000 ml
Dilute stock solution 1/7 and use as tank buffer. Follow the same precautions as before to avoid corrosion of electrodes by $MgCl_2$.

(5) 0.05M phosphate/0.007M citric acid buffer pH 7.0
Di-sodium hydrogen orthophosphate
(anhydrous) (Na_2HPO_4) 7.097 g
Citric acid (2-hydroxy-propane
1,2,3 tricarboxylic acid) 1.471 g
Water (distilled) to 1000 ml

B.15.2 Spectrophotometric Assay Buffers and Developer Buffers

(1) 0.3M Tris/HCl buffer pH 8.0

Tris (hydroxymethyl) aminomethane		36.34 g
HCl (1.0M)		42.00 ml
H_2O (distilled)	to	1000 ml

(2) 0.3M Tris/HCl buffer pH 7.4

Tris (hydroxymethyl) aminomethane		36.34 g
HCl (1.0M)		63.00 ml
H_2O (distilled)	to	1000 ml

B.16 Conditions Affecting the Mobilities of the Enzymes

In order to obtain optimum resolution of the isoenzymes of T. vaginalis, conditions of growth, lysate preparation, storage and electrophoresis were varied. The various conditions were as follows:

B.16.1 Variation in preparation of T. vaginalis:

(a) After cloning, the same organisms of the same stock were cultivated in two or more different kinds of media. These media were: modified Lumsden medium (Ackers et al., 1975), Stenton's medium (Stenton, 1957) and Hollander's fluid medium (Hollander, 1976).

(b) Two different cultures of the same stock of T. vaginalis in the same medium.

(c) Isolated flagellates from hamsters infected with T. vaginalis (the Beckenham stock). The human T. vaginalis stock (No. 34870) originally used to infect the hamster intravaginally; this will be known as the Beckenham parent.

(d) A trichomonad isolated from the faeces of monkeys at London Zoo, this organism will be known as the monkey strain.

(e) Organisms of T. vaginalis from Italy, kindly supplied by Professor Cappuccinelli. These organisms were compared with organisms of T. vaginalis isolated from London.

(f) Pentatrichomonas hominis from American Type Culture Collection (AC 30000). This organism was compared with organisms of T. vaginalis

B.16.2 Treatment of Trichomonas extracts:

Comparisons were made between some extracts of T. vaginalis as follows:

(a) After preliminary electrophoresis, lysates showing differences in mobilities were mixed together and compared with the original one.

(b) Lysates of some stocks stored at -196°C were compared after different periods of storage, e.g. 2 months, 4 months and 8 months.

B.16.3 Variation in electrophoretic conditions:

(a) Buffer composition;

(b) Buffer pH/molarity;

(c) Duration of electrophoretic run;

(d) Voltage.

As a result of this work, optimum conditions for the assay of trichomonad enzymes were established. These conditions are shown in Table 10. Table 11 shows the developing conditions found to give the best results.

B.17 Procedure for Concanavalin A-induced Agglutination

The method described by Trissl et al. (1977) was followed but with some modification. Stocks of T. vaginalis, flagellates from monkeys and hamsters (the Beckenham and monkey stocks) and the Beckenham parent were used. Three different media for culture of organisms were used throughout these experiments. T. vaginalis was grown in modified Lumsden's medium, flagellates from monkeys were grown in Robinson medium (Robinson, 1968) with reduced rice starch. The Beckenham stock and Beckenham parent were grown in Hollander's medium (Hollander, 1976) with and without agar. All Trichomonas organisms were cultivated axenically and passaged twice before they were harvested for these experiments. Organisms were harvested, washed twice by centrifugation at 200 g for 10 minutes in pBS (pH 7.2) and were resuspended in fresh PBS so that the final concentration of organisms was 1 - 2 x 10^6/ml. Experience showed that using a concentration of organisms of more than 1 x 10^6/ml facilitates immediate reaction and observation of any agglutination. Using either microtitre agglutination plates or migration inhibitory plates (Nunc, Intermed, Denmark) 150 µl of Trichomonas suspension were mixed with equal volume of concavalin A (Con A) (Sigma) of known

concentration in PBS (pH 7.2) and incubated for 30 minutes at 37°C with intermittent shaking of the plates.

Final concentrations of Concanavalin A used were 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml.

The plates were examined directly under microscope at x 10 magnification and confirmed by examination of a drop of the precipitate on a clean glass-slide at x 40 magnification.

The size of Trichomonas clumps was measured using a calibrated eye-piece at x 10 magnification when the number of Trichomonas forming clumps was difficult to estimate, otherwise, the degree of clumping was estimated by counting the average number of the cells per clump in 3 experiments. Final concentration of Con A below which no agglutination occur was considered as end point.

The specificity of the reaction was tested by pre-incubating an equal volume of double-strength concavalin A with 0.2M α-methyl mannoside (Sigma) to abolish the agglutination reaction.

A control was set up using PBS alone to exclude spontaneous agglutination which may occur in certain strains.

B.18 Growth Rate and Generation Time

A technique for growth rate measurement based on the time elapsing before the population density reaches an arbitrary (low) value; which has been used for T. vaginalis (Kulda et al., 1970); for Plasmodium berghei (Warhurst and Flowell, 1968); and for Entamoeba histolytica (Farris, 1978) was adopted for culture of T. vaginalis. Groups of 5 ml culture bottles were inoculated with serial dilutions of an inoculum of T. vaginalis. In all instances the original inocula were taken from 48 hours cultures in the same medium. Counts of living T. vaginalis were made with the aid of haemocytometer and trypan-blue (0.2%) exclusion. The counts were made at least in duplicate. In order to get an accurate estimate of the time for the culture to reach 20,000 organisms/ml (pre 20,000 time) for each inoculum when sampling daily at the same time, two consecutive daily measurements were chosen which were above and below 20,000 organisms/ml; these figures were plotted against elapsed time. The value of pre-20,000 time was then estimated by interpolation on a straight line drawn between the two values. The number of days taken for each culture to produce a fixed low number of organisms (20,000) should be linearly related to the log of the inoculum size providing growth rate remains constant (Figures 20 a and b).

PART III

RESULTS

C. RESULTS

The results of this work are divided into:

Section A: Isolation and cultivation of specimens.

Section B: Light microscopy

Section C: Electron microscopy

Section D: Clones

Section E: Isoenzyme electrophoresis

Section F: Concanavalin A induced agglutination

Section G: Growth rate and generation time.

C.I Section A: Isolation and cultivation of specimens

C.1.1 Isolation of T. vaginalis

Assembly of Hemming's filter used in collection of vaginal secretion can be seen in Figure 21. Generally the weight of vaginal secretion from patients infected with T. vaginalis is slightly higher than those from patients not infected with T. vaginalis (Tables 20, 21).

C.1.2 Cultivation of T. vaginalis

Strains of T. vaginalis used throughout this work (listed in Tables 14-16 & 26) grew in modified Lumsden's medium MLM (Ackers et al., 1975), modified Diamond's TYM medium (Hollander and Frost, 1965) and in Stenton's medium (1957).

Growth was found to be more rapid, and the number of organisms ultimately harvestable was larger in MLM than in the other media. In MLM growth reaches the stationary phase at pH 5, and it is hard to find viable organisms below pH 4.3. The change in colour of the medium to yellow (indicating production of acids) can help in screening of culture bottles. However, it was found that not all culture bottles showing yellow colour were positive for T. vaginalis. Some yellow cultures were found to contain only Candida or bacteria. In most cases, cultures became positive the day after inoculation with vaginal secretion infected with T. vaginalis, others took several days (up to 7 days) before they became positive. Some specimens found negative for T. vaginalis on wet film examination, were positive on inoculation in culture bottles. In no case did we find negative culture bottles after inoculation with samples positive by wet film examination.

The harvesting of T. vaginalis by centrifugation from MLM was easier than from modified Diamond's TYM medium, as the agar present in the latter was often deposited with the organisms. Preliminary experiments showed that T. vaginalis does not grow well in modified Diamond's TYM medium without agar.

C.1.3 Isolation of flagellates from monkey faeces

Flagellates isolated from faeces of monkey

were found to grow only in Robinson's (1968a) medium. Attempts to isolate these flagellates in MLM with or without added rice starch and with or without saline-agar slopes were unsuccessful.

C.1.4 Isolation of Beckenham (Hamster) strain

This strain was found to grow in Robinson's medium and in Stenton's medium but not in MLM. The parent of the Beckenham strain and the mouse-virulent strains (from Italy) were found to grow better in MLM than in Robinson's or modified Diamond's TYM medium.

C.2 Section B: Light Microscopy

C.2.1 T. vaginalis

Using the modified Giemsa staining method of Neva et al. (1961) the morphology of T. vaginalis is well shown (Figures 22-27). This method of staining was found to show morphological details such as the number of anterior four flagella, the recurrent flagellum and undulating membrane, the nucleus and the axostyle. The conditions under which Giemsa is usually used (diluted 1:6 to 1:10 for up to 30 minutes) causes severe overstaining of T. vaginalis and little if any detail is visible. Different outline contours of T. vaginalis were seen in the stained

preparation of different samples and even among populations of the same sample. Photograph of dividing forms of T. vaginalis with two or more than two nuclei are shown in Figures 25-27,29-30. In these cases it appears that, either each nucleus goes to each pole of the organism (Figure 26) or both separating nuclei remain at one pole (Figure 27). Apparently, separation takes place either transversally or longitudinally. Abnormal (round) forms of T. vaginalis (which we prefer to call "giant forms") are occasionally seen in cultures; they are shown in Figures 28,29, 30. These "giant forms" are not artefacts but can be seen in living populations as round, stationary forms, apparently with flagella not beating but adherent to the body. Multinucleate abnormal forms we also present (Figures 29, 30). No attempt has been carried to separate these forms for future studies.

C.2.2 Flagellates isolated from monkey faeces

All the three flagellates isolated from monkey faecal specimens showed the same morphology. This flagellate was found to possess three anterior flagella and a recurrent flagellum with undulating membrane which extended beyond the anterior half of the body (Figures 31,32,33, and 34). The size of this organism ranged from 10.3 to 11.6 µm in length and from 8.2 to 10.9 µm in width with

average of 10.5 µm and 9 µm respectively. The three anterior flagella are nearly equal in normal nondividing organisms with length ranging from 12.3 to 13.7 µm with an average of 13 µm. More morphological details are shown in the results of ultrastructure studies. Starch granules were found to be necessary for growth of this flagellate in Robinson's medium but omitting Es. coli from the medium showed that growth of this flagellate did not depend on the presence of bacteria. We have been able to grow this flagellate axenically without difficulty. The avid engulfing of starch granules by this flagellate is interesting and can be seen in Figures 31-34 and 41, 46.

C.2.3 Beckenham (hamster) strain

The light microscopic studies of this organism, both directly from the hamster vagina and from culture in Stenton's medium showed the same morphology as T. vaginalis except that (in a sample obtained directly from a hamster) the prominent undulating membrane could be clearly seen to extend more than half way down the body, while the recurrent flagellum continued beyond the end of the membrane (Figures 35-40). The size of the specimen from hamster vagina (8.8 ± 1 × 6.4 ± 1.1 µm) was found to be smaller than that grown in Stenton's medium (9.6 ± 1.3 × 8.3 ± 2 µm) (Figures 37 - 40). The parent of the Beckenham strain was morphologically similar to the rest of the T. vaginalis stocks. The significance of these findings will be discussed later.

C.3 Section C: Electron Microscopy

Because of our wish to identify the flagellate isolated from monkey faeces, it was necessary to study the ultrastructure of this parasite. Figures 41, 42, 46 are electron micrographs of the whole flagellate cut at different levels. In the upper organism in Figure 41 costa, the cisternae of the parabasal body (Golgi apparatus), the three anterior flagella and the recurrent flagellum can be seen; in the lower organism the three kinetosomes of the three anterior flagella, the kinetosomes of the recurrent flagellum, the axostyle, electron-dense granules (probably hydrogenosomes) and the nucleus may be seen. The axostyle and the nucleus with surrounding endoplasmic reticulum are shown in Figure 42. In Figure 46 two very large starch granules which have been engulfed by the parasite are visible. The fin-like cytoplasmic extension which forms the undulating membrane (with marginal lamella) is seen in Figure 43 , and a cross section of the undulating membrane can be seen in Figure 44 ; note that at this point the recurrent flagellum appears not to be connected to the fin-like cytoplasmic extension. A similar gap has been observed in Trichomonas gallinae (Figure 29 in Hongiberg, 1978a and Figure 40 in Mattern et al., 1973). The three anterior flagella in the periflagellar canal (the relatively shallow depression in the anterior surface of the

organism) are shown in Figure 45. Figure 47 shows the costa and a parabasal fibre. Details of costa (Type A) are shown in Figure 48. The pattern of such a costa consists of multilinear repeating units (Honigberg, 1978a), and each unit possesses 4 bands (Brugerolle, 1976). A row of paracostal granules (probably hydrogenosomes) parallel to the costa can also be seen. Details of the kinetosomes of the anterior flagella, rootlet filaments, parabasal fibre, granules and costa are seen in Figure 49 In the same plate, a periodic comb-like organelle can be seen. Figure 5I (at higher magnification) is of the origin of the locomotive system and shows parabasal filaments, rootlet filaments and the kinetosome of recurrent flagellum between the kinetosomes of the anterior flagella.

C.4 Section D: Clone

Hollander's (1976) method of growing T. vaginalis in solidified modified Diamond's TYM medium was found in our hands the best way to obtain colonies of T. vaginalis and other trichomonads. Colonies of T. vaginalis in agar inoculated with different suspensions are shown in Figures 51, 52 and 53. Colonies were confluent when suspensions of higher concentrations were used. Despite the relative softness of the agar, Hollander's method was found to be the best and most reliable for cloning of T. vaginalis. It was found, from comparing the size of the inoculum and the number of colonies obtained, that the plating efficiency was more than 75% (Table 13). In our hands the micro-

manipulation methods which we tried for cloning (see Materials and Methods) were unsuccessful and no growth from single organisms was obtained.

C.5 Section E: Isoenzyme Electrophoresis

Soluble extracts from T. vaginalis stocks and other trichomonads were subjected to horizontal thin-layer starch-gel electrophoresis and were specifically stained for the following enzymes:

(1) Aldolase (ALD)
(2) Malic enzyme (oxaloacetate decarboxylating) (ME)
(3) Lactate dehydrogenase (LDH)
(4) Glucose phosphate isomerase (GPI)
(5) Phosphoglucomutase (PGM)
(6) Malate dehydrogenase (NAD^+ oxireductase) (MDH)
(7) Hexokinase (HK)
(8) Glucose-6-phosphate dehydrogenase (G6PD).

These producedures were repeated three times for each enzyme for every stock in order to confirm the original results.

Generally, the viability of cells used in the preparation of extracts (lysates) was at least 90%. No variations were found in the electrophoretic patterns when any one or combination of the following were varied:

(a) age of culture,
(b) medium used in cultivation,

(c) activity of extracts (lysates) before electrophoresis (Figures 58, 59).

Media used for preparation of extracts (lysates) in these experiments were:

(a) modified Lumsden medium for:
- T. vaginalis
- Italian mouse-virulent strains
- parent of Beckenham (hamster) strain;

(b) Robinson's medium for:
- flagellates from faeces of monkey;

(c) Stenton's medium for:
- Beckenham (hamster) strain
- Pentatrichomonas hominis.

Once the optimum conditions for electrophoresis had been determined, the average volume of extract required to test one enzyme was found to be 5 µl. When extracts of T. vaginalis stocks with different patterns were mixed together and subjected again to electrophoresis, complex staining patterns were obtained as expected (Figures 71,77,98). On omitting the substrate from the developer, bands of enzyme activity were never seen; such control plates were always run in parallel with the experimental plates (Figure 73).

C.5.1 Variation in electrophoretic conditions

Preliminary experiments showed that the composition of the tank buffer, the buffer pH and molarity and

running voltage affected the appearance of bands on the starch-gel for each enzyme [see Figures 55 (bowing due to high voltage); 64,65 (different buffers)]. The optimum electrophoretic conditions are summarized in Table 10. Our results showed no difference in the intensity of staining of the electrophoretic bands when the crude extracts were compared before and after storage in liquid nitrogen (-196°C) for approximately one and a half years. The effect of prolonged subculture on electrophoretic pattern has not been investigated.

C.5.2 Spectrophotometric assays

C.5.2.1 Protein

To standardize the protein assay, a calibration curve was used in every time if the whole specimens not been screened at once. The results of protein estimation, using bovine serum albumen as a standard are shown in Table 17-19. The calibration curves are shown in Figures 1 - 3 and the protein content of different samples are summarized in Tables 14-16.

C.5.2.2 Enzyme analysis

Enzyme activities of different extracts were assayed according to the procedure of Lanham and Scott (personal communication). The specific activities of

three enzymes (HK, MDH and LDH) for thirty stocks of samples are summarized in Table 12 . Curves to show how time and absorbance units are read off the curves can be seen in Figure 15 . From the data presented in Table 12 , it can be seen that the specific activities of the individual enzymes varied significantly. In general, hexokinase (HK) activity was found higher than malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) and even vary among different isolates of T. vaginalis. However, three extracts (mouse-virulent strain from Italy (1192 & 1193) and T. vaginalis (76/714(Tv1)) showed relatively high MDH activity.

C.5.3 Electrophoretic pattern of enzymes

The results of electrophoresis of the enzymes examined are summarized in Figures 8,9,10,11,12,13,14. In most cases, the enzyme patterns appear within five minutes after specific staining and rapidly become diffuse on further incubation. The enzyme patterns are classified into:

C.5.4 Enzymes for which electrophoretic patterns were similar

C.5.4.1 Aldolase (ALD)

(1) T. vaginalis

Each extract showed five separate, sharp, intense bands (Figures 54-59 and 62). The pattern

of this enzyme was found to be constant even on slightly changing of the electrophoretic conditions. The mouse-virulent strains showed the same pattern as T. vaginalis.

(2) Monkey flagellates

Extracts of this flagellate showed one clear band which developed more slowly than those of T. vaginalis (Figure 62). No difference in electrophoretic pattern could be seen among the three different strains isolated from monkey faeces.

(3) Beckenham (hamster) strain

Attempts to locate bands of aldolase activity on the gel for this strain were unsuccessful, while the pattern of the parent stock was identical to normal T. vaginalis. The inability to locate such bands will be discussed below.

C.5.4.2 Malic enzyme (oxaloacetate decarboxylating ($NADP^+$)(ME))

(1) T. vaginalis

The electrophoretic pattern of different strains of T. vaginalis, mouse-virulent strains and the parent of Beckenham (hamster) strain can be seen in Figures 60, 61 and 63 and are summarized in Figure 9 . Each extract showed three bands with a weak (fastest) one which developed later than the other two. Diffusion from the origin is characteristic of zymograms of this particular enzyme (see below).

of this enzyme was found to be constant even on slightly changing of the electrophoretic conditions. The mouse-virulent strains showed the same pattern as T. vaginalis.

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(2) Beckenham (hamster) strain and monkey isolates

Attempts to locate bands of enzyme activity in these strains were unsuccessful.

C.5.5 Enzymes showing differences between T. vaginalis strains

C.5.5.1 Lactate dehydrogenase (LDH)

Different systems to locate bands of activity for this enzyme were tried as seen in Figures 64 and 65. The optimum conditions of electrophoresis for this enzyme can be seen in Table 10.

(1) T. vaginalis (including mouse-virulent strains)

The electrophoretic pattern of different extracts can be seen in Figures 66 - 71. Four different patterns among different extracts of T. vaginalis were observed and these are:

- LDH I: pattern with four regular, sharp, intense bands A, B, C and D. Examples can be seen in samples number 13,14,15,18 and 20 in Figures 67, 68, 69 and 70.

- LDH II: pattern with two sharp, intense bands corresponding to bands A and C in pattern I. Examples can be seen in Figures 66, 67 and 69 (samples numbers 17, 21, 22, 33 and possibly 8).

- LDH III: pattern with two sharp intense bands corresponding to bands B and D in pattern I (Figure 69 and samples numbers 25, 26, 27 and 29).

- LDH IV: pattern with two sharp, intense bands corresponding to bands C and D in pattern I (Figures 66, 67, 68 and samples numbers 1, 2, 6, 16, 19, 31).

(2) Monkey flagellate and Beckenham (hamster) strains

Extracts of these isolates did not show any zymogram pattern for LDH as shown in Figure 71. while extract of parent Beckenham strain grown in two different media showed the LDH I pattern (Figure 71).

C.5.5.2 Glucose phosphate isomerase (GPI)

(1) T. vaginalis

Using this enzyme, the electrophoretic pattern of different extracts examined showed bands which diffuse shortly after development. T. vaginalis stocks usually gave a pattern with three diffuse bands (A, B and C) or one with band C missing (Figures 74 - 77). However, one extract (mouse-virulent strain number 1192) showed band A missing, while two other extracts (T. vaginalis number 6 and 16) showed an extra, slower band (Figure 74).

(2) Monkey flagellates

The electrophoretic pattern of GPI of three different isolates of flagellates from monkey faeces showed in considerable differences from those of T. vaginalis (Figures 74 and 77). Among the monkey strains, one (M3) showed a pattern different from

M1 and M2, characterized by the presence of a second slower anodal band (Figures 74 and 77).

(3) Beckenham (hamster) strain

The electrophoretic pattern for GPI of this strain showed two bands which were faster than any given by T. vaginalis (Figures 76 and 77).
The parent of the Beckenham strain (grown in two different media) showed the pattern with bands A and B given by several strains of T. vaginalis (Figure 77).

C.5.5.3 Phosphoglucomutase (PGM)

(1) T. vaginalis

All extracts of T. vaginalis strains showed the same electrophoretic pattern with single band which was always in the same position and diffused within five minutes after development (Figures 78, 79, 80, 81, 82). Only one extract (mouse-virulent strain number 1192) gave a band relatively faster than all the others.

(2) Monkey flagellates

The electrophoretic pattern of two extracts (M1 and M2) showed split bands located at the level of the relatively faster band of the mouse-virulent strain number 1192. The third extract (M3) showed two slower bands and one faster band when compared with the other two extracts (M1 and M2) (Figure 80).

(3) Beckenham (hamster) strain

The electrophoretic pattern showed two faster bands located in different positions when compared either with those of T. vaginalis or with monkey strains. The parent of the Beckenham (hamster) strain (grown in modified Lumsden medium or Stenton's medium) showed a pattern which was similar to that of the other T. vaginalis strains (Figure 82).

C.5.5.4 Malate dehydrogenase (MDH)

(1) T. vaginalis

Using this enzyme, different extracts of T. vaginalis showed three different patterns (Figures 83, 84,85,86,87,88,89,90,91) which are:

- MDH I: pattern with three bands A, B and C.

- MDH II: pattern with two bands corresponding to bands A and B in MDH I.

- MDH III: pattern with two bands corresponding to bands B and C in MDH I.

It may be seen that of the two Italian mouse-virulent strains 1192 showed MDH III pattern while the other one number 1193 showed MDH I pattern (Figures 83, 84, 85).

(2) Monkey flagellates

Two different strains (M1 and M2) showed the same pattern with three intense anodal bands located

near to the origin and different from those of T. vaginalis. The third strain (M3) showed a pattern with two faster different from the other two (M1 and M2) and those of T. vaginalis (Figures 83, 84, 85 and 88).

(3) Beckenham (hamster) strain

The electrophoretic pattern was different from all the other samples (Figures 83, 84, 85 and 88). The parent of the Beckenham strain showed the MDH I pattern.

C.5.5.5 Hexokinase (HK)

It was particularly noticeable with this enzyme that the position of the sample in the plate affected the electrophoretic pattern. The best resolution was given when the origin of the samples was midway between cathode and anode (Figures 92 and 93).

(1) T. vaginalis

Two electrophoretic patterns of different strains of T. vaginalis were found (Figures 93, 94, 95, 96, 97, 98) they are:

- HK I: pattern with two intense bands (A and B) and third one weak band (C).

- HK II: pattern with two intense bands corresponding to bands A and B in HK I pattern, but no band C. The mouse-virulent strain number 1192 showed the HK I pattern while strain number 1193 showed the HK II pattern.

(2) Monkey flagellates

The three different monkey stocks showed the same pattern: a single intense band faster than any of those of T. vaginalis (Figures 94 and 95).

(3)Beckenham (hamster) strain

While the extract of this strain did not show electrophoretic pattern for HK, the parent extract showed HK I pattern as those of T. vaginalis.

Electrophoretic patterns for the enzymes tested of P.hominis are different from the other trichomonads and can be seen in Figures 9, 11, 12 and 14.

C.6 Section F: Concanavalin A-induced Agglutination

The results of concanavalin A-induced agglutination are shown in Figures 99 - 104. All T. vaginalis showed agglutination with con A at a final concentration of 100 µg/ml. When different concentrations of con A (less than 100 µg/ml) were used, different strains showed different values for the minimum concentration causing agglutination (see Table 22). According to isoenzyme grouping the results of con A agglutination may be summarized as:

Group I : no agglutination occurs below 12.5 µg/ml final concentration of con A.

Group II : no agglutination occurred below 25 µg/ml final concentration of con A.

Group III & IV : no agglutination below 50 µg/ml final concentration of con A.

Group V : no agglutination below 100 µg/ml final concentration of con A.

The hamster strain did not show agglutination by con A (at 100 µg/ml) while the parent showed agglutination at 12.5 µg/ml final concentration of con A.

One monkey strain (M3) did not show agglutination at 100 µg/ml con A while the other two (M1 and M2) showed agglutination at 25 µg/ml con A.

The Italian strains (M92 and 1193) showed agglutination with con A at 50 µg/ml and 12.5 µg/ml respectively.

In all cases no spontaneous agglutination of the parasites occurred in the absence of the lectin during the course of the experiments. Agglutination did not occur with con A when inhibitory concentrations of α-methyl-D-mannoside was present.

C.7 Section G: Growth Rate and Generation Time

The results of generation time (G) of T. vaginalis are shown in Table 22 and summarized in Figure 19. According to these results, T. vaginalis can be classified into three groups:

Group A : characterized by slow growth and with G greater than 11 hours.

Group B : characterized by medium growth and with G between 6 and 11 hours.

Group C : characterized with fast growth and with G less than 6 hours.

According to isoenzyme patterns, strains of Group I were found with long G; strains of Groups II, III and IV were found with medium G, and strains of Group V were found with short G. The hamster strain showed a short generation time while the parent showed a long generation time. The Italian strains (1192 and 1193) showed medium and long G respectively. One of the monkey strains (M1) showed long G while the other two (M2 and M3) were short.

PART IV

DISCUSSION
AND CONCLUSIONS

D. DISCUSSION AND CONCLUSIONS

D.1 Isolation and Culture Medium

We have shown that culture techniques for the isolation of T. vaginalis are more sensitive and more accurate for diagnosis than simple examination of a wet film of vaginal exudate. During collection of our samples, 22% of positive cases for T. vaginalis were only found using this technique. The formulae of some of the many media which have been proposed for the cultivation of Trichomonas are summarized in Taylor and Baker (1968 and 1978); most are complex and undefined. This makes them difficult to manipulate experimentally in order to study the effect of single components. Modified Lumsden medium (MLM; Ackers et al., 1975) was found to be convenient for the culture of T. vaginalis; it is easy to prepare and sterilize and supports growth from a small inoculum. Using this medium, the colour change to yellow (indicating the production of acids) can save time when many culture bottles are used. Microscopic examination is necessary to check culture bottles because some yellow cultures were found to contain only Candida and/or bacteria. In most cases, cultures became positive 24-48 hours after inoculation with vaginal secretions containing T. vaginalis, others took several days (up to 7) before they became positive. This might

be due to differences in the number, viability and generation time of inoculated organisms.

Honigberg (1978b) reported that media containing agar are better than those of comparable constitution that lack the agar. This was true in our case only for cloning, otherwise T. vaginalis was found to grow better in MLM which lacks agar, than in modified Diamond's TYM medium, which contains 0.25% agar (Hollander and Frost, 1965). The use of an agar-containing medium was particularly undesirable for our work, since the agar is deposited when organisms are harvested by centrifugation.

Although outside the scope of this work, it has proved possible to prepare from the same sample of vaginal contents, a supernatant for antibody assay (Ackers et al., 1975) and to cultivate T. vaginalis and Lactobacillus acidophillus from the sediment.

It is difficult to know the significance of the observation of a slight increase in the weight of vaginal secretion from patients infected with T. vaginalis. Whether this is specific to trichomoniasis or applies in all vaginal infections is not yet clear.

Axenic culture is necessary for many investigations, but the disadvantages should also be recognized. Kott and Adler (1961) found that two strains of T. vaginalis of different antigenic types became identical after the culture has been bacterially contaminated and then cleaned with antibiotics. There is no problem in obtaining axenic culture of T. vaginalis since the

introduction of penicillin (Adler and Pulvertaft, 1944) and streptomycin (Quisno and Foter, 1946), but it is recommended (Honigberg, 1978b) that antibiotics should not be incorporated routinely in culture media used for the maintenance of trichomonads by serial passage. It was claimed that streptomycin caused changes in the pathogenicity of T. gallinae (Stabler et al., 1964 and Honigberg et al., 1970) a species closely related to T. vaginalis. However, serial passage in vitro can be avoided by using cryopreservation and we ensured that all cultures used in this work were no more than five subcultures from original isolation. Because all bacteria were not necessarily dead by this time, penicillin and streptomycin were routinely included in all cultures. All cultures were routinely checked microscopically and any containing visible bacteria were discarded.

Despite the excellence of MLM for T. vaginalis attempts to use this medium to isolate other trichomonads were unsuccessful. Robinson's (1968a) medium was found to be the only medium suitable for isolation of flagellates from faeces of monkeys. As far as is known the presence of Es. coli in Robinson's medium is necessary for the growth of E. histolytica. Omitting the bacteria did not prevent the growth of flagellates from faeces of monkeys and axenic culture could be easily obtained. As with E. histolytica, rice starch granules were found to be necessary for growth of this flagellate. The Beckenham

(hamster) strains can be grown in Robinson's medium and in Stenton's (1957) medium but not in MLM. These differences among media suitable for the growth of these different trichomonads may reflect inherent metabolic characteristics of these parasites. In general, the optimum pH for all media was found to be 6.6 - 6.8 except for Stenton's medium which was pH 6.0; the optimum incubation temperature was 37°C for all the different trichomonads used in this work. Medium which had been kept at -20°C for three months supported growth but to a lesser degree than fresh (up to two weeks old) medium. It was unfortunate that all the organisms used could not be grown in the same medium. However, growth in different media was found not to affect the isoenzyme patterns (see below). As far as is known, enzymes (as genetic markers) are not changed by as simple a procedure as growth for short period in a different medium. The fact that not all organisms would grow in the same medium provided more evidence to support the specific biological characters of certain strains. For instance, while the Beckenham (hamster) strain failed to grow in MLM, the parent grew very well in this medium, while the flagellate isolated from monkey faeces grew only in Robinson's medium.

D.2 Light Microscopy

D.2.1 T. vaginalis

The method of fixation and staining of Neva et al. (1961) was found to be reproducible and more reliable than the conventional Giemsa staining method. Some strains appear, on average, rounder than others although individual variations can be seen among members of a single population. However the size and shape of T. vaginalis are affected by the fixation method and our observations are in agreement with Abraham and Honigberg (1964), Glebski (1969a) Honigberg and King (1964) and Wenrich (1939). Another explanation for these variations in the tendency of T. vaginalis to attach to surface of solid objects (Lumsden et al., 1966; Samuels, 1961). All strains have the capacity to form pseudopodia-like extensions. Because of these variations, it is not satisfactory to use such measurements to differentiate species of Trichomonadinae on the bases of size and shape. A further problem concerns the position of the dividing nuclei in stained preparation of T. vaginalis. It appears that separation takes place either transversally or longitudinally across the body of the parasite. However, further investigations would be needed to understand the reason for this variation.

D.2.2 Abnormal and round forms

The existence of a cyst stage during the life cycle of T. vaginalis has been a subject of controversy, and recently was a major topic of discussion at the International Symposium on Genitourinary Trichomoniasis, Paris (1977). On light microscopic examination of living specimens, our observation of round, motionless forms of T. vaginalis were in agreement with previous investigators (e.g. Trevaux et al., 1978). However, further details of these organisms (such as flagella and nucleus) could be seen using the modified method of Giemsa staining. No distinguishable cyst membrane could be seen in these forms; this is clearly visible in Amoeba or Giardia cysts. It is known that in old culture or under adverse conditions, dying T. vaginalis cells tend to be rounded; however these dying forms are different from the round forms which we are observed and which are capable of multiplication as seen in the stained preparation. We refer to such large forms as "giant forms". To the best of my knowledge this is the first detailed report of these round forms of T. vaginalis in stained preparation. It seems likely that these are the forms which might previously have been described as cysts or pseudocysts, particularly when these forms are seen in living preparations without beating flagella (which apparently adhere to the body of the parasite).

However, further investigations would be needed to know the pathological significance of these forms, and our opinion (in agreement with many others) is that cysts are not formed by T. vaginalis and that the parasite exists only in the trophozoite form.

D.2.3 Beckenham (hamster) strain

This strain was supplied originally as a strain of T. vaginalis passaged into vagina of golden hamster. Light microscopic study of stained preparation both from hamster's vagina and culture in Stenton's medium has shown this strain with four anterior flagella. The fifth recurrent flagellum was found trailing beyond the end of the undulating membrane, which extends beyond the anterior half of the body. The parent of this strain (the human isolate which was adapted to grow in the hamster vagina) was found to show the typical morphology of T. vaginalis. In the view of this information and other results which will be discussed later, it is apparent that the Beckenham (hamster) strain which we isolated from hamster's vagina is different from (and may not be) the original parent strain. It is possible that this train is an intestinal Trichomonas from the hamster gut, accidentally transferred to the vagina although the possibility of a genuine change cannot be dismissed. However, apart from morphological differences, the unsuccessful attempts to grow Beckenham strain in MLM in which the parent grows well, could also

support the suggestion that this is not T. vaginalis. This will be discussed later in the section concerning isoenzyme electrophoresis.

D.2.4. Flagellates from faeces of monkey

The three isolates from faeces of monkey were found to be very similar morphologically. They had three anterior flagella and a recurrent flagellum carrying the undulating membrane which extends beyond the anterior half of the body. Similar light microscopy studies have been reported by Wenrich and Nie (1949) and Wenrich (1944a). Wenrich (1944a) and Kessel (1928a, cited by Wenrich, 1944a) found two kinds of Trichomonas in the intenstine of different monkeys. One of these possessed three anterior free flagella and the other was found to have four. However, apart from the two papers by Wenrich (1944a) and Wenrich and Nie (1949) there is little detailed published work on intestinal trichomonads from faeces of monkey. Brumpt (1909a) and Levine (1973) devoted only a paragraph each to the subject. This flagellate was studied by light microscopy, electron microscopy and isoenzyme electrophoresis. Under light microscopy these flagellates were found to be highly active in engulfing starch granules. Brumpt (1909) reported an interesting observation in a monkey with dysentery. On histological examination, he found considerable numbers of Entamoeba and Trichomonas. He

claimed that mucomembranous inflammation was due to Trichomonas and not to Entamoeba which proved later to be E. coli and non-pathogenic when inoculated intrarectally into four cats. To the best of our knowledge, this observation passed unnoticed. The question of association of intestinal trichomonad and Entamoeba and whether or not this relationship could aggravate amoebiasis deserve attention. Using light microscopy it seems that these flagellates from the faeces of monkey most closely resemble a Trichomitus-spp. It is reported by Levine (1973) that such kinds of flagellates occur in the caecum and colon of rhesus monkey and chacma baboon.

D.3. Electron Microscopy

For the purpose of our work, the aim of electron microscopy was to identify flagellates isolated from faeces of monkey. Electron microscopy allows us to see in greater detail structure discernible with the light microscope, and in addition it provides for resolution of some structures whose details cannot be seen by one of the known light microscopic techniques especially the kinetosomes. Because of scarcity of published electron micrographs of Trichomitus, our interpretation came from comparison mainly with electron micrographs published by Honigberg et al. (1972), Mattern et al. (1973), Honigberg (1978a) and Brugerolle (1976). It is evident from the descriptive account that these flagellates share

with Trichomonadinae most of its fine-structural characteristics especially those pertaining to the undulating membrane. The undulating membrane consists of high body fold, enclosing the loop-shaped marginal lamella and with the recurrent flagellum applied to the fold. At certain level of section, the membrane of the recurrent flagellum appears not to be connected to the fin-like dorsal cytoplasmic extension. A similar gap has been observed in this section of the undulating membrane of Trichomonas gallinae (Figure 29, p. 176 in Honigberg, 1978a). This monkey flagellate has also certain structures (the costa and comb-like organelles) in common with Trichomitus-type organism published by Honigberg et al. (1972). Of interest is the observation of a Type A costa; the pattern of such costa consisting of multilinear repeating units. In the light of this information gathered suggestion that these flagellates are Trichomitus-spp. would be supported.

D.4 Cryopreservation

For the purpose of our work cryopreservation was necessary. On prolonged serial passage, the biological characters of microorganisms may be changed. For instance, prolonged in vitro cultivation of T. vaginalis and closely related organisms may cause significant loss of virulence (Dwyer and Honigberg, 1970; Honigberg et al., 1970; Laan, 1966; Lindgren and Ivey, 1964; Stabler

et al., 1964). Many investigators thought that the virulence might be conserved by viable preservation of the organisms by freezing. By cryopreservation the biological characters are not fundamentally altered, and the properties of individual samples of stabilate (given impeccable conditions of cryopreservation) remain constant for periods of at least several years. For instance, Diamond et al. (1965) found T. vaginalis retained its original virulence after two years' storage in liquid nitrogen (-196°C). Similar findings have been made with T. gallinae (Honigberg et al., 1970) which retained its original virulence to pigeons after 5, 5.5 and 7 years of storage. Recently, the results of Ivey (1975) indicated that freezing per se does not affect the virulence of recent isolates of T. vaginalis and most importantly, the original virulence of such isolates can be preserved for long periods of time by storage in liquid nitrogen. For the purpose of our work and in view of the previous findings, it was necessary to preserve our samples in liquid nitrogen. The procedure described by Lumsden et al. (1966) for low temperature preservation of T. vaginalis and Lumsden et al. (1973) for trypanosomes were adopted using 8% (v/v) final concentration of dimethylsulphoxide (DMSO) as cryoprotectant. It is important to differentiate between stabilates and strains. According to Lumsden and Hardy (1965), the term strain is to indicate material

maintained available by serial passage in culture. Strains are designated according to the patients from which they were isolated. The term stabilate is used as suggested by Lumsden and Hardy (1965) to indicate viably preserved material in which reproduction is arrested and selection (except for that associated with the process of preservation) thereby avoided. A stock is a population derived by serial passage *in vitro* and/or *in vivo* from a primary isolation with or without any implication of homogeneity or characterization (WHO, 1978). This word has replaced the word "strain" which is more generally used. In our experience almost all stabilates retrieved successfully. However, failure to retrieve some stabilates might be due to manipulation during cryopreservation and to a low count of organism preserved.

D.5 Clones

Without special precaution, *T. vaginalis* strains and stabilates may be genetically heterogeneous, because they may contain representatives of several different populations existing in the original isolates. Even cultures of a single species are likely to be genetically heterogeneous (Farris, 1978a). However, for biochemical, physiological and other studies, it is desirable to work with clones. Pure populations of *T. vaginalis* have been

successfully obtained by growing them up from single organisms (Magara et al., 1953; Asami et al., 1955; Samuels et al., 1960; Samuels, 1962; Cavier et al., 1964; Hollander et al., 1965; Ivey, 1961, 1975a and b; and Hollander,1976). Ivey (1975a) recommended that clones can be used by biologists to study the effect of other factors on cell viability particularly effect of freezing procedure variations. Such recommendation is beyond the scope of our work but was important in Farris' (1978a and b) work in which she used micromanipulation method for preparing clone culture of E. histolytica which used for study of the factors which determine the survival of this organism during cryopreservation. With Trypanosoma and Leishmania spps. clones have been widely used to study mixed infection using isoenzyme techniques (e.g. Kilgour, 1976; Al-Taqui, 1978). In fact the question of mixed infection in trichomoniasis and interpretation of this in regard to clinical manifestation, immunological and biological studies, constitute fruitful areas for future studies. For the purpose of our work, it is necessary to work with "clones" because deriving a population from a single organism minimizes deviation of the biological characteristics. Micromanipulation methods in liquid medium or in agar medium are difficult and time consuming because of the need to search for a single viable organism, the need for two colleagues to confirm the presence of this single organism before inoculation, and the high risk

of contamination during manipulation. In our hands, these methods were unsuccessful. However, the results of cloning using micromanipulations were found to differ among different investigators. For instance, Müller (personal communication) found it very difficult to obtain clones of T. vaginalis in his laboratory, while Honigberg (personal communication) using a special adaptor to pick up single organisms successfully obtained clones of T. vaginalis. Apparently application of such method in T. vaginalis needs experience and should be carried out quickly to avoid adverse conditions. In our hands the pour-plate method of Hollander (1976) for preparing cloned cultures of T. vaginalis was easy, successful and overcame the disadvantages of previously mentioned method. Strict anaerobic conditions for incubated plates are necessary and McIntosh and Filde's anaerobic jar is suitable and successful. Discrete spherical colonies develop even in very crowded plates and proportionate numbers of colonies develop even when the inoculum is very dilute. Sensitivity of such method was more than 75%. Recently, Wilson and Ackers (1980) using this method were able to enumerate small numbers of organisms in assessing value of urine cultures in diagnosis of trichomoniasis in males.

D.6 Isoenzyme Electrophoresis

D.7 Spectrophotometry assay

The occurrence and variation in specific activity of many enzymes of T. vaginalis were reported by Aresa et al. (1974), Teras et al. (1973), Baernstein (1961, 1959). Using a spectrophotometric assay, Teras et al. (1973) divided 25 strains of T. vaginalis into groups with high, medium and low hexokinase activity. The results of the three enzyme assays [hexokinase (HK), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH)] of thirty T. vaginalis strains in the present work generally support the observations by previous workers. The activity differences among T. vaginalis strains are very interesting, but it is not possible to state whether or not these differences reflect true metabolic differences because the nature of the solublization procedure did not ensure disruption of all organelles. Another variable is the quantity of the stabilizer solution used for lysis of the organisms. This was not measured, so the concentration of the enzymes will vary somewhat from preparation to preparation of the same stock. However, since the major emphasis of this study was on qualitative rather than quantitative aspects, the spectrophotometric analysis merely served as a guide to concentration of enzymes for electrophoresis of the crude extracts.

D.8 Identification of trichomonads by isoenzyme electrophoresis

Characterization of kinetoplastid flagellates by means of isoenzyme variation has shown that it is possible to distinguish between species of Leishmania (Kilgour et al., 1974; Kilgour, 1976; Al-Taqui and Evans, 1978; Gardner and Howells, 1972; Gardner et al., Ebert, 1973 and 1974), Entamoeba (Reeves et al., 1968; Montalvo et al., 1968; Sargeaunt et al., 1978a and b, 1979), Plasmodium (Carter and McGregor, 1973; Carter and Voller, 1973 and 1979), Trypanosoma (Miles et al., 1977, 1978; Godfrey, 1975, 1977, 1978; Godfrey et al., 1976; Kilgour et al., 1973, 1975; Kilgour, 1976; Bagester and Parr, 1973; Gibson, 1977; Gibson et al., 1978). In the present work, isoenzyme analysis by means of thin-layer starch-gel electrophoresis has been shown to be promising for the characterization of trichomonads. It is a relatively simple technique and highly reproducible. It is important, however, that the isoenzyme pattern of several enzymes are examined when different strains of different trichomonads are being compared. In general, the isoenzyme patterns of all enzymes tested in this work showed considerable differences between different trichomonad species which we used. Godfrey (1975) claimed that biochemical methods could now offer objective definition of subspecies. Among the three strains of Trichomitus-type organisms isolated

from the faeces of monkeys, two strains were easily distinguished by their characteristic patterns from the third one suggesting the presence of at least two different groups within this species. More groups may be found in the future on testing more samples.

In regard to T. vaginalis, the fact that all strains show similar isoenzyme patterns of certain enzymes [for example aldolase (ALD) and malic enzyme (ME)] should not be regarded as a disadvantage; rather it tends to give confidence in the close similarities between members of the same species. We would not agree with Musisi (1979) that these enzymes are not worth examining. These isoenzymes can be as important for identification as those isoenzymes which show clear electrophoretic differences.

It seemed at first that the isoenzyme patterns of phosphoglucomutase (PGM) would be similar in all T. vaginalis strains tested; however, one of the two Italian strains (Lump 1192) showed a faster band. It is therefore possible that this enzyme will divide T. vaginalis strains into two groups, but further testing will be necessary to confirm this.

It was not possible from the work done so far to explain the failure of some strains to show any bands at all with some enzymes. Failure could have been due to lack of activity, unsatisfactory conditions for the development, or because, in T. vaginalis and other trichomonad tested, the enzyme is unusually labile. Another

possibility is the relatively crude extracts used might not be suitable for testing such enzymes. Our results with glucose-6-phosphate dehydrogenase (G6PD) would be supported in view of the previous suggestion. Using this enzyme, while some strains of T. vaginalis showed diffuse patterns, many others did not show any patterns. Similar difficulties in obtaining clear patterns using G6PD have been reported by Miles et al. (1977) using Trypanosoma cruzi. Other enzymes which were tried in preliminary experiments and found not to work at all were alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT). There are no published reports of the presence of these enzymes in trichomonads although they are present in other protozoa (for example Trypanosoma and Leishmania) and were used successfully by Kilgour (1976).

D.9 Differences in isoenzyme patterns among T. vaginalis strains

Among eight satisfactory enzymes in the present study, lactate dehydrogenase (LDH) seemed very promising. The four different isoenzyme patterns (I, II, III and IV) obtained using this enzyme, divided T. vaginalis strains into four groups. It is interesting that Teras divided his isolates into four groups also on the basis of antigenic type; it has not yet proved possible to see if the two classifications coincide.

With glucose phosphate isomerase (GPI), the electrophoretic patterns obtained from most strains of T. vaginalis fell mainly into two groups. There is a possibility, however, of two further groups existing because one of the two Italian strains (Lump 1192) showed a missing band and another strain (No. 16) showed an extra slower band which is not present in the other groups.

Malate dehydrogenase (Oxaloacetate $NADH^+$) (MDH) is another enzyme which showed promising and reproducible results. It is possible to divide T. vaginalis strains into three different groups according to the electrophoretic pattern using this enzyme. However, more groups might appear in the future when more samples are tested. This suggestion is made because two forms (isoenzymes) of malate dehydrogenase (MDH) of one strain of T. vaginalis were isolated by Brugerrolle et al. (1973) using sucrose density gradient and polyacrylamide gel electrophoresis. One form was found located in the soluble fraction and the other is linked to the cytoplasmic "dense granules". Although these authors have not compared different T. vaginalis strains, the photograph published by them showing electrophoretic pattern of the soluble form of MDH, was similar to one of the three patterns which we found.

Using hexokinase enzyme (HK), we obtained two different electrophoretic patterns dividing our T. vaginalis strains into two groups. Comparison of these two groups

with the three groups obtained by Teras et al. (1973) is difficult at this stage, because they divided T. vaginalis strains with high, medium and low activity. Using this method of grouping, they were able to correlate their results with virulence but not antigenic differences among the 25 strains they used.

D.10 Beckenham (hamster) strain and parent

Electrophoresis of the enzymes from these two organisms showed considerable differences between the patterns found. While the electrophoretic patterns of enzymes tested of the parent organisms were similar to normal T. vaginalis strains, the patterns of the Beckenham (hamster) strain were quite different. As discussed before, the morphology of Beckenham (hamster) strain was found to be different from that of the parent. It is of course possible that growth in a different host might affect the isoenzyme patterns. However, Miles et al. (1977) reported that it is highly improbable that the enzymic differences were influenced by the different species of original host, and Kilgour (1975) observed that isoenzyme patterns in different hosts were the same. Similar observations were also reported by Al-Taqui (1978) in the same Leishmania stocks from different hosts and from different sites of infection. In the present study, these observations by previous workers are supported by the results obtained using the two Italian strains. No

differences were observed in this study between the patterns from cells harvested after different periods of culture. It therefore seems unlikely that the differences in the electrophoretic patterns were due to switching on or off of certain isoenzymes at different developmental stages of the cells. It is possible that certain constituents of the media used in cultivation could affect the metabolism of the organisms, and the possibility exists that different quantities of isoenzymes are produced in response to the presence or absence of different nutrients. However, in this study the media did not affect the isoenzyme patterns of strains cultivated in two media (Stenton's medium and modified Lumsden medium). The results of this work using different conditions of electrophoresis showed that the composition of the buffer solution (tank, gel and developer), molarity/pH, voltage, distance of origin between cathode and anode (in HK) could affect the rate of mobility and separation of isoenzymes. In addition, the developer solution which contains the specific substrate and dye also could affect the appearance and the intensity of the enzyme banding. However in practice there is no doubt that the complex isoenzyme patterns obtained from two extracts of T. vaginalis mixed together and subjected to electrophoresis, were the sum of separate pattern of each strain.

D.11 Significance of isoenzyme electrophoresis in trichomonad

While the significance of the present work cannot be wholly assessed until more is known about the nature of multimolecular forms, it can at least be said that there are now different markers, which it is reasonable to assume are genetic markers, to identify subpopulations of *T. vaginalis* and to distinguish them from *Trichomitus*-type organisms in particular and different trichomonads in general. The enzymes tested in this work are unlikely to be the only markers and others can be looked for with some confidence. The method of identification offers the beginning of a biochemical classification to supplement the present morphological, immunological and pathological classifications. With a biochemical classification a pattern may appear which could relate factors in the organism to other characteristics, and in time not only a number of objective features but those that are significant may emerge.

D.12 Concanavalin A

Concanavalin A induced agglutination has been demonstrated by many investigators to be useful tool in the study of surface properties of cells to differentiate between pathogenic (or virulent) and nonpathogenic (or avirulent) organisms. We applied such technique in attempts to study the surface properties of T. vaginalis, looking for differences among T. vaginalis strains. The results of concanavalin A suggest that T. vaginalis has specific polysaccharides (glycoprotein) residue on its external surface. Agglutination of T. vaginalis with con A was specific and occurred only when the lectin was present; it was inhibited when con A was incubated with α-methyl-D-mannoside. As we suggested before, different strains of T. vaginalis which showed different isoenzyme patterns might be different in other biological properties such as differences in cell surface. Although all T. vaginalis were agglutinated by con A at 100 µg/ml, strains showed differences in the minimum concentration of con A necessary to cause agglutination. This may well be due to differences in the amount and distribution of saccharides binding to con A on the surface of strains of T. vaginalis; however this needs further investigation. Another less likely possibility is that changes in agglutinability are due to alteration of cell surface properties due to differring rates of division, since there is a strong correlation with generation time (see

below). Such possibility would also need further investigation. Only two trichomonads showed no agglutination with 100 µg/ml con A in this study: one monkey strain (M3) and the Beckenham (hamster) strain. The result of con A induced agglutination shows that is a very promising technique particularly since the results correlate so well with isoenzyme patterns (Table 22 and Figure 19).

D.13 Generation Time

In a further attempt to seek for correlation between isoenzyme variations and other biological properties, the estimation of generation time G was undertaken. According to the generation time, T. vaginalis strains may be divided into long (slow growth), medium, and short (fast growth) groups. There was strong correlation between these groups and different isoenzyme patterns (Figure 19). Kulda et al. (1970) indicated an apparently high degree of positive correlation between the generation time of a strain in vitro and its pathogenicity as tested by the mouse subcutaneous assay. They found strains of long generation time to be more pathogenic than the others. Further studies would be needed to confirm these correlations which were not readily appeared from our examination of clinical data. Using the pre-20,000 method, the generation time (G) in days was calculated by the standard formula:

$$G = \frac{t \cdot \log 2}{\log N' - \log N}$$

where t = time elapsed in days

N' = population after time t

N = initial population.

If N' = 20,000, N = initial inoculum and t = pre 20,000 time, then if log inoculum is plotted against pre 20,000 time, G = - log 2/slope

No record is available concerning the cytopathological changes that accompanied infection with T.vaginalis. The very strong correlation amongst T.vaginalis isolates between isoenzyme, con A and growth rate strongest support the reality of the groups we formed.

From all the evidence the organism which we isolated from the hamster's vagina (and which was the only one which could be successfully passaged in this site (Ackers, unpublished) is not T.vaginalis. Morphologically it appears to resemble a Tetratrichomonas spp. more closely than other Trichomonadinae but without authentic specimen to compare with we cannot be more definite than that. Interestingly, Levine (1973), lists amongst other member of the genus Tetratrichomonas, Tet. macacovaginae from the vagina of rhesus monkey, and Tet. microti from the caecum of many rodents including the hamster. There seems no doubt that the organism originally to infect the hamster vagina (the Beckenham parent) is a perfectly conventional isolate of T. vaginalis, it seems most likely that at some later date it was replaced by a different species but the matter is still under investigation.

Table 8: Enzymes mobilities studied by electrophoresis

Recommended name of enzyme and reaction	Systematic name of enzyme	Enzyme commission no.	Abbreviation
Glucose phosphate isomerase Glucose-6-phosphate ⇌ fructose-6-phosphate	D-glucose-6-phosphate ketolisomerase	E.C.5.3.1.9	GPI
Phosphoglucomutase (G1:6 diP) α-D-glucose-1-phosphate ⇌ α-D-glucose-6-phosphate	D-glucose-1,6-biphosphate: D-glucose-1-phosphate phosphotransferase	E.C.2.7.5.1	PGM
Malic enzyme L-Malate + $NADP^+$ → Pyruvate + CO_2 + $NADPH^+$	L-Malate : $NADP^+$ oxireductase (oxaloacetate decarboxylating)	E.C.1.1.40	ME
Hexokinase D-glucose + ATP^+ → D-glucose-6-phosphate + ADP^+	ATP^+ : D-glucose-6-phosphotransferase	E.C.2.7.1.1	HK
Malate dehydrogenase L-Malate + NAD^+ ⇌ Oxaloacetate + $NADH^+$	L-Malate : NAD^+ oxireductase	E.C.1.1.1.37	MDH
Glucose-6-phosphate dehydrogenase D-glucose-6-phosphate + $NADP^+$ → 6-phosphogluconate + $NADPH^+$	D-glucose-6-phosphate : NAD^+ 1-oxireductase	E.C.1.1.1.49	G6PD
Aldolase Fructose 1,6-diphosphate ⇌ dihydroxyacetone phosphate + D-glyceraldehyde-3-phosphate	Fructose-biphosphate aldolase	E.C.4.1.2.13	ALD
Lactate dehydrogenase L-Lactate + NAD ⇌ Pyruvate + NADH	L-lactate : NAD^+ oxireductase	E.C.1.1.1.27	LDH

Table 9: Enzymes for which total activity was measured

Enzyme Commission number	Recommended name of enzyme	Systematic name of enzyme	Abbreviation
E.C.2.7.1.1	Hexokinase	ATP^+ : D-glucose-6-phosphotransferase	HK
E.C.1.1.1.37	Malate dehydrogenase	L-Malate : NAD^+ oxireductase	MDH
E.C.1.1.1.27	Lactate dehydrogenase	L-Lactate : NAD^+ oxireductase	LDH

Table 10: Optimum conditions for enzyme electrophoresis

Enzyme	Tank Buffer (1 litre)	pH	Volts cm^{-1} across gel	Current (mA)	Gel buffer made from tank buffer	Running time (hours)
GPI	0.2M Na-phosphate	7.0	16	15	1/13	2.5
PGM	0.1M Tris/malate	7.4	16	15	1/10	2.5
ME	0.2M Na-phosphate	7.0	16	15	1/10	3
HK	Stock: 0.9M Tris/0.2M EDTA/0.02M Boric acid/0.025M $MgCl_2$ Tank: dilute 1/7	8.6	20	≈20	1/10	3.5
MDH	0.05M phosphate/0.007M citric acid	7.0	20	≈20	1/13	3
G6PD	0.2M Na-phosphate	7.0	16	15	1/10	3
ALD	0.1M Na-phosphate	7.3	20	≈20	1/10	3.5
LDH	0.1M Tris/malate	7.8	20	≈20	1/5	3

The temperature of the cooling plate was kept at $0^{o}C$ throughout the run.

Table 11: Composition of enzyme developers

Enzyme	Developing conditions (conc. in the final volume) Buffer	Substrate	Coenzyme	Other conditions	$MgCl_2$ 0.1M	MTT	PMS	Agar L28, 12 mg/ml
GPI	0.3M Tris/HCl pH 8, 10 ml	10 mg/ml fructose-6-phosphate, 1 ml	10 mg/ml $NADP^+$ (4-Na-salt), 0.5 ml	100 U/ml G-6-PD solution, 0.5 ml	0.5 ml	2 mg	1 mg	10 ml
PGM	0.3M Tris/HCl pH 7.4, 10 ml	25 mg glucose-1-phosphate with 1% G-1,6-diP.	10 mg/ml $NADP^+$ (4-Na-salt), 0.4 ml	100 U/ml G-6-PD solution, 150 µl	0.5 ml	2 mg	1 mg	10 ml
HK	0.1M Tris/HCl pH 7.4, 10 ml	-450 mg D-glucose -60 mg ATP di-sodium salt	25 mg $NADP^+$ (4-Na-salt)	100 U/ml G-6-PD, solution 10 µl	0.5 ml	4 mg	2 mg	10 ml
ME	0.3M Tris/HCl pH 7.4, 8 ml	Malate solution* 1 ml	10 mg/ml $NADP^+$ (4-Na-salt), 0.5 ml	-	2 ml	5 mg	2 mg	10 ml
MDH	0.3M Tris/HCl pH 8, 6 ml	Malate solution, 4 ml	10 mg/ml HAD^+ (di-sodium salt), 1 ml	-	-	2 mg	1 mg	10 ml
LDH	0.05M Tris/HCl pH 8, 10 ml	Ca-lactate (Sigma), 35 mg	10 mg/ml NAD^+ (di-Na-salt), 25 mg	-	-	5 mg	2 mg	10 ml
ALD	0.3M Tris/HCl pH 8, 6 ml	-Fl,6 DP, 72 mg -glyceraldehyde-3-phosphate dehydrogenase (G-3PD), 96 units	10 mg/ml NAD^+ (di-Na-salt), 0.72 ml	-0.3M Na-arsenate 1 ml $-H_2O$, 2.03 ml	-	2 mg	1 mg	10 ml
G-6PD	0.2M Tris/HCl pH 8, 7 ml	-glucose-6-phosphate (G6-P), 5 mg	10 mg/ml $NADP^+$ (4-Na-salt), 2 mg	-	3 ml	2 mg	1 mg	10 ml

* Malate solution: 10 mg/ml L-malic acid adjusted to pH 7.0 with 1.0M NaOH.

Table 12: Protein content and enzyme activity for various T. vaginalis

Specimen	Code	Protein content mg/ml	HK		MDH		LDH	
			Enzyme units ml^{-1}	Specific activity µ/mg. protein	Enzyme units ml^{-1}	Specific activity µ/mg. protein	Enzyme units ml^{-1}	Specific activity µ/mg. protein
76/714	TV1	4.3	0.181	0.042	2.964	0.689	0.373	0.087
Lump 994	TV2	10.3	2.431	0.236	0.155	0.015	2.487	0.241
Lump 996	TV3	6.5	1.352	0.208	0.878	0.139	0.487	0.077
75/7479	TV4	22.0	0.704	0.032	2.635	0.119	3.544	0.161
75/7940	TV5	10.9	0.392	0.036	0.504	0.046	2.984	0.274
75/8213	TV6	14.0	3.010	0.215	2.289	0.164	0.011	0.001
76/754	TV7	14.0	1.946	0.139	0.784	0.056	1.190	0.085
75/8403	TV8	9.7	0.412	0.042	-	-	0.071	0.007
Lump 1314 (76/30)	TV9	16.0	2.473	0.155	5.290	0.331	-	-
Lump 1315	TV10	13.0	2.138	0.164	1.069	0.082	0.445	0.034
75/8555	TV11	-	1.466	-	-	-	0.121	-
76/991	TV12	12.0	2.619	0.218	0.065	0.005	0.270	0.023
76/2002	TV13	13.0	2.028	0.156	3.120	0.240	0.117	0.009
76/415	TV14	13.3	1.237	0.093	0.971	0.073	1.357	0.102
76/7689	TV15	12.0	1.656	0.138	1.392	0.116	0.816	0.068
Lump 1242	TV16	14.0	2.814	0.201	0.532	0.038	1.204	0.086
75/2040	TV17	18.0	4.320	0.240	1.440	0.080	0.414	0.023
75/8095	TV18	15.8	3.808	0.241	0.458	0.029	0.458	0.029
Lump 1160	TV19	13.0	2.379	0.183	1.209	0.093	1.339	0.103
Lump 1165	TV20	12.5	2.413	0.193	0.972	0.078	1.500	0.120
Lump 1064	TV21	14.0	1.806	0.129	1.414	0.101	1.820	0.130
Lump 1065	TV22	13.7	8.990	0.656	0.466	0.034	1.836	0.134
Lump 1192 (Italy)	TV23	9.4	0.802	0.085	2.063	0.219	0.357	0.038
Lump 1193 (Italy)	TV24	6.3	0.588	0.093	1.603	0.254	0.243	0.039
yt/434	TV25	5.9	0.321	0.054	1.912	0.324	0.780	0.132
Lump 1042	TV26	16.0	8.496	0.531	8.880	0.555	6.912	0.432
Lump 1043	TV27	14.0	0.207	0.015	4.494	0.321	8.694	0.621
Lump 1044	TV28	4.4	0.160	0.036	0.149	0.034	0.343	0.078
Lump 1031	TV29	12.0	1.824	0.152	1.704	0.142	1.416	0.118
Lump 1046	TV30	4.0	2.364	0.591	1.281	0.304	0.594	0.149

Table 13: Results of agar plating. Average number of colonies were compared with number of of organisms added to each plate.

Number of *T. vaginalis* added to each plate	Number of plates becoming positive	Average number of colonies per plate (%)
1000	3/3	880 (88)
500	3/3	350 (70)
100	3/3	75 (75)
50	3/3	35 (70)

Table 14: Results of protein estimation (Folin-Ciacalteau) of lysates of T. vaginalis. Absorbance at wavelength 660 nm.

Specimen	Code	Absorbance	Average	Protein concentration from standard curve (Fig.1) mg/ml	Final results (reading from standard curve x dilution of sample) mg/ml
75/714	TV1	0.267 0.267	0.267	0.43	4.3
Lump 994	TV2	0.543 0.728	0.6355	1.03	10.3
Lump 996	TV3	0.381 0.389	0.3895	0.65	6.5
75/7479	TV4	0.146 0.143	0.1445	0.22	22.0
75/7940	TV5	0.693 0.653	0.673	1.09	10.9
75/8213	TV6	0.086 0.093	0.0895	0.14	14.0
75/757	TV7	0.067 0.100	0.0835	0.14	14.0
75/8403	TV8	0.590 0.618	0.604	0.97	9.7
Lump 1314 (76/30)	TV9	0.099 0.102	0.1005	0.16	16.0
Lump 1315	TV10	0.080 0.075	0.0775	0.13	13.0

Table 15: Results of protein estimation (Folin-Ciacalteau) of lysates of T. vaginalis. Absorbance at 660 nm wavelength.

Specimen No.	Code No.	Absorbance	Average	Protein concentration from standard curve (Fig.2) mg/ml	Final results (reading from standard curve x dilution of sample) mg/ml
76/991	TV12	1.192 1.278	1.235	1.2	12.0
76/2002	TV13	1.404 1.192	1.298	1.3	13.0
76/415	TV14	1.432 1.222	1.327	1.33	13.3
76/7689	TV15	1.394 1.092	1.243	1.2	12.0
Lump 1242	TV16	1.242 1.652	1.447	1.4	14.0
75/2040	TV17	1.776 1.916	1.846	1.8	18.0
75/8095	TV18	1.714 1.416	1.565	1.58	15.8
Lump 1160	TV19	1.178 1.372	1.275	1.3	13.0
Lump 1165	TV20	1.170 1.326	1.248	1.25	12.5
Lump 1064	TV21	1.166 1.714	1.440	1.4	14.0
Lump 1065	TV22	1.134 1.598	1.366	1.37	13.7

Table 16: Results of protein estimation (Folin-Ciacalteau) of lysates of T. vaginalis and monkey flagellates. Absorbance at 660 nm wavelength.

Specimen No.	Code No.	Absorbance	Average	Protein concentration from standard curve (Fig.3) mg/ml	Final result (reading from standard curve x dilution of sample) mg/ml
Lump 1192 (Italy)	TV23	0.939 0.934	0.9365	0.94	9.4
Lump 1193 (Italy)	TV24	0.649 0.623	0.636	0.63	6.3
76/434	TV25	0.597	0.597	0.59	5.9
Lump 1042	TV26	1.186 1.183	1.162	1.60	16.0
Lump 1043	TV27	1.374 1.434	1.404	1.40	14.0
Lump 1044	TV28	0.392 0.487	0.4395	0.44	4.4
Lump 1031	TV29	1.308 1.092	1.200	1.20	12.0
Lump 1046	TV30	0.396 0.401	0.3985	0.40	4.0
P/2243 (monkey)	M1	0.340 0.331	0.3355	0.33	3.3
SAW206 (monkey)	M2	0.358	0.358	0.35	3.5
P/2384 (monkey)	M3	0.277 0.258	0.2675	0.25	2.5

Table 17: Results of Protein estimation (Folin-Ciacalteau) of the standard sample (Bovine serum albumen). Absorbance at 660 nm wavelength.

Protein concentration of the standard sample	Absorbance	Total of Absorbance	Average of absorbance
0.1 mg/ml	0.167	0.321	0.1605
	0.154		
0.25 mg/ml	0.355	0.731	0.3655
	0.376		
0.5 mg/ml	0.643	1.259	0.6295
	0.616		
0.75 mg/ml	0.886	1.764	0.882
	0.878		
1.0 mg/ml	1.060	2.118	1.059
	1.058		

Table 17: Results of Protein estimation (Folin-Ciacalteau) of the standard sample (Bovine serum albumen). Absorbance at 660 nm wavelength.

Protein concentration of the standard sample	Absorbance	Total of Absorbance	Average of absorbance
0.1 mg/ml	0.167	0.321	0.1605
	0.154		
0.25 mg/ml	0.355	0.731	0.3655
	0.376		
0.5 mg/ml	0.643	1.259	0.6295
	0.616		
0.75 mg/ml	0.886	1.764	0.882
	0.878		
1.0 mg/ml	1.060	2.118	1.059
	1.058		

Table 18: Results of protein estimation (Folin-Ciacalteau) of the standard sample (bovine serum albumen). Absorbance at 660 nm wavelength.

Protein concentration of the standard sample	Absorbance	Total of Absorbance	Average of Absorbance
0.1 mg/ml	0.128	0.256	0.128
	0.128		
0.25 mg/ml	0.308	0.616	0.308
	0.308		
0.5 mg/ml	0.664	1.328	0.664
	0.664		
0.75 mg/ml	0.683	1.357	0.6785
	0.674		
1.0 mg/ml	1.028	2.036	1.018
	1.008		
Blank	0.004	0.032	0.016
	0.028		

Table 19: Results of protein estimation (Folin-Ciacalteau) of the standard sample (bovine serum albumen). Absorbance at wavelength 660 nm.

Protein concentration of the standard sample	Absorbance	Total of Absorbance	Average of Absorbance
0.1 mg/ml	0.068	0.129	0.0645
	0.061		
0.25 mg/ml	0.167	0.320	0.160
	0.153		
0.5 mg/ml	0.287	0.597	0.2985
	0.310		
0.75 mg/ml	0.425	0.837	0.4185
	0.412		
1.0 mg/ml	0.560	1.229	0.6145
	0.669		

Table 20: Random cases not infected with *T. vaginalis*.

Specimen No.	Weight dry	Weight + secretion	Weight of secretion	Volume of secretion
75/7368	19.7503	19.9956	0.2453	0.7 ml
75/7370	19.7500	19.8431	0.0931	0.7 ml
75/7371	19.7272	20.0703	0.3431	0.8 ml
75/7372	19.5660	19.6357	0.697	0.8 ml
75/7373	19.6436	19.6770	0.0334	0.6 ml
75/7374	19.8150	20.0911	0.2761	0.7 ml
75/7376	19.8128	20.4606	0.6478	0.6 ml
75/7823	20.5773	20.7855	0.2082	0.6 ml
75/7827	19.7083	20.6149	0.9066	0.8 ml
75/7830	20.1110	20.5080	0.397	0.6 ml
75/7831	20.3019	20.4302	0.1283	0.8 ml
75/7832	19.4119	19.6801	0.2682	0.8 ml
75/7835	19.7404	20.0428	0.3024	0.7 ml
75/7837	19.7547	19.9263	0.1716	0.7 ml
75/7840	19.8857	19.8887	0.0030	0.75 ml
75/7842	19.9136	20.5013	0.5877	0.95 ml
75/8032	18.7902	19.0457	0.2555	0.75 ml
75/8034	19.8957	20.0868	0.1911	0.75 ml
75/8035	20.6564	20.9273	0.2709	0.65 ml
75/8037	20.6393	20.7900	0.1507	0.85 ml
75/8166	19.9435	19.0490	0.1055	0.60 ml
75/8167	21.1133	20.3628	0.2495	0.95 ml
75/8178	19.1462	19.4380	0.2918	0.70 ml
75/8179	17.7721	17.9723	0.2002	0.80 ml
75/8181	20.0050	20.0599	0.0594	0.80 ml
75/8183	19.8316	20.1650	0.3334	0.70 ml
75/8186	21.1469	20.2916	0.1447	0.70 ml
75/8191	19.5923	19.6988	0.1065	0.60 ml

Table 21: Cases infected with T. vaginalis.

Specimen No.	Weight dry	Weight + secretion	Weight of secretion	Volume of secretion	Remarks
75/7268	19.6889	19.9245	0.2356	0.95 ml	
75/7269	19.8550	20.2344	0.3794	0.90 ml	
75/7349	19.7720	19.9687	0.1967	0.80 ml	
75/7357	19.8344	20.0739	0.2395	0.60 ml	
75/7377	19.8282	20.0143	0.1861	0.90 ml	
75/7479	19.8268	20.8993	1.0725	1.05 ml	
75/7689	19.7614	20.0118	0.2504	0.90 ml	
75/7940	19.6151	19.7131	0.0980	0.45 ml	+ve after culture
75/8040	20.4295	20.6748	0.2453	0.90 ml	
75/8043	18.7670	19.6701	0.9031	0.80 ml	+ve after culture
75/8095	20.6282	21.0214	0.3932	0.80 ml	+ve after culture
75/8098	20.5169	20.5701	0.0532	0.80 ml	
75/8213	20.8570	21.1550	0.2980	0.90 ml	
75/8217	20.8973	21.3749	0.4776	1.00 ml	
75/8251	20.5392	20.7709	0.2317	0.80 ml	+ve after culture
75/8403	20.7575	20.8520	0.0945	-	
75/8449	18.7670	19.7010	0.9340	0.70 ml	+ve after culture
75/8555	20.3392	21.8688	1.5296	1.30 ml	
75/8584	21.0678	21.1662	0.0984	0.60 ml	+ve after culture
75/8761	20.9904	21.4214	0.4310	0.75 ml	
75/8817	20.4573	21.2894	0.8321	0.80 ml	
76/30 (L 1314)	20.6190	20.8834	0.2644	0.85 ml	
76/136	20.4561	20.8049	0.3488	0.75 ml	
76/415	19.7237	19.8874	0.1637	0.75 ml	
76/434	19.2229	19.5320	0.3091	0.90 ml	+ve after culture
76/714	20.9187	21.3519	0.4332	0.80 ml	
76/754	20.5813	20.7752	0.1939	0.85 ml	+ve after culture
76/991	18.5455	18.5845	0.0390	0.80 ml	
76/1206	20.3820	20.6628	0.2808	1.00 ml	
76/1232	20.3505	20.6036	0.2531	0.80 ml	
76/1235	19.9587	20.1807	0.222	0.95 ml	
76/1264	20.9686	21.4158	0.4472	0.90 ml	
76/1265	20.3472	20.4933	0.1461	0.50 ml	
76/2002	21.5639	22.3995	0.8356	1.00 ml	
76/2044	19.7986	20.1361	0.3375	0.80 ml	
76/2071	19.9740	20.6869	0.7129	0.80 ml	
76/2075	20.7680	20.9587	0.1907	0.85 ml	
76/2205	19.6534	19.8295	0.1761	0.75 ml	
76/2217	19.6262	19.6846	0.0584	-	
76/2418	19.7968	19.8943	0.0975	0.70 ml	
76/2512	19.1169	19.2127	0.0958	0.90 ml	
76/2535	19.4015	19.5537	0.1522	0.65 ml	
76/2598	21.0654	21.9005	0.8351	0.85 ml	

Table 22: Results of generation time and concanavalin A-induced agglutination of *T. vaginalis*.

Specimen No.	Code	Generation time (hours)	Minimum Concentration of Con A (µg/ml)
76/714	TV1	6.50	50.0
Lump 994	TV2	7.10	50.0
Lump 996	TV3	3.35	100.0
75/7479	TV4	13.00	12.5
75/7940	TV5	12.00	12.5
75/8213	TV6	8.00	50.0
76/754	TV7	3.72	100.0
75/8403	TV8	9.00	50.0
Lump 1314 (76/30)	TV9	12.00	12.5
Lump 1315	TV10	6.30	25.0
75/8555	TV11	11.30	12.5
76/991	TV12	7.20	25.0
76/2002	TV13	11.30	12.5
76/415	TV14	11.45	12.5
76/7689	TV15	12.30	12.5
Lump 1242	TV16	7.00	25.0
75/2040	TV17	6.30	50.0
75/8095	TV18	12.00	12.5
Lump 1160	TV19	10.00	50.0
Lump 1165	TV20	14.00	12.5
Lump 1064	TV21	7.20	50.0
Lump 1065	TV22	8.00	50.0
Lump 1192) Italy	TV23	9.00	50.0
Lump 1193)	TV24	14.00	12.5
76/434	TV25	8.55	25.0
Lump 1042	TV26	6.30	25.0
Lump 1043	TV27	9.00	25.0
Lump 1044	TV28	7.50	50.0
Lump 1031	TV29	8.00	25.0
Lump 1046	TV30	4.00	100.0
Lump 1264	TV31	10.00	50.0
Lump 1232	TV32	3.00	100.0
75/8043	TV33	6.70	50.0
75/8040	TV34	12.45	12.5
Beckenham (hamster) strain		5.00	-
Beckenham parent strain		11.45	12.5

Fig. 1

Standard curve of protein estimation from table(17).

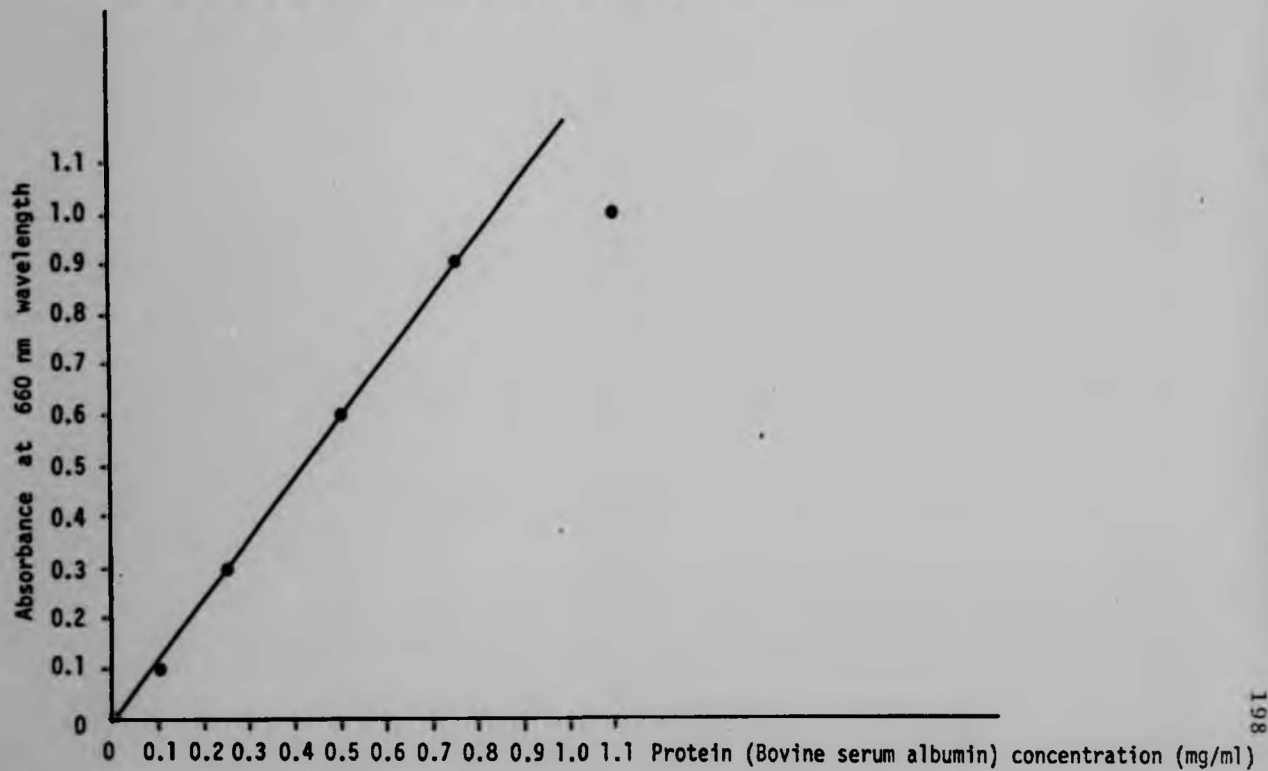


Fig. 2

Standard curve of protein estimation from table (18).

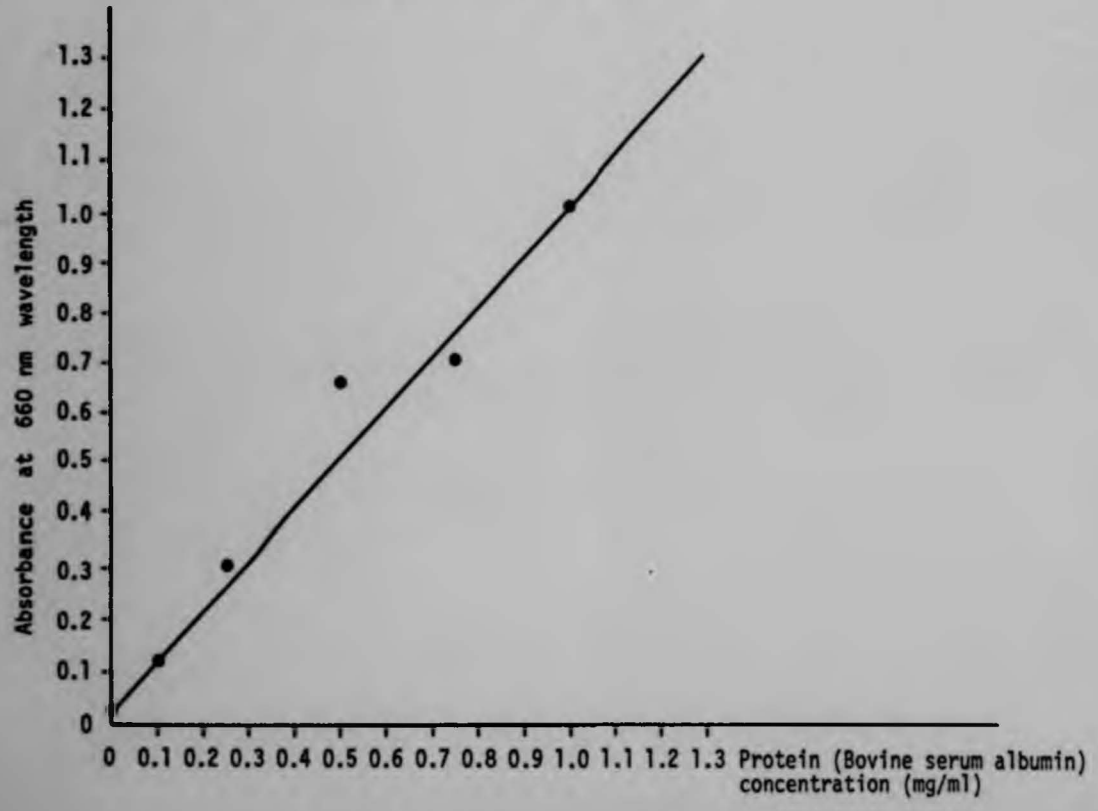


Fig. 3

Standard curve of protein estimation from table (19).

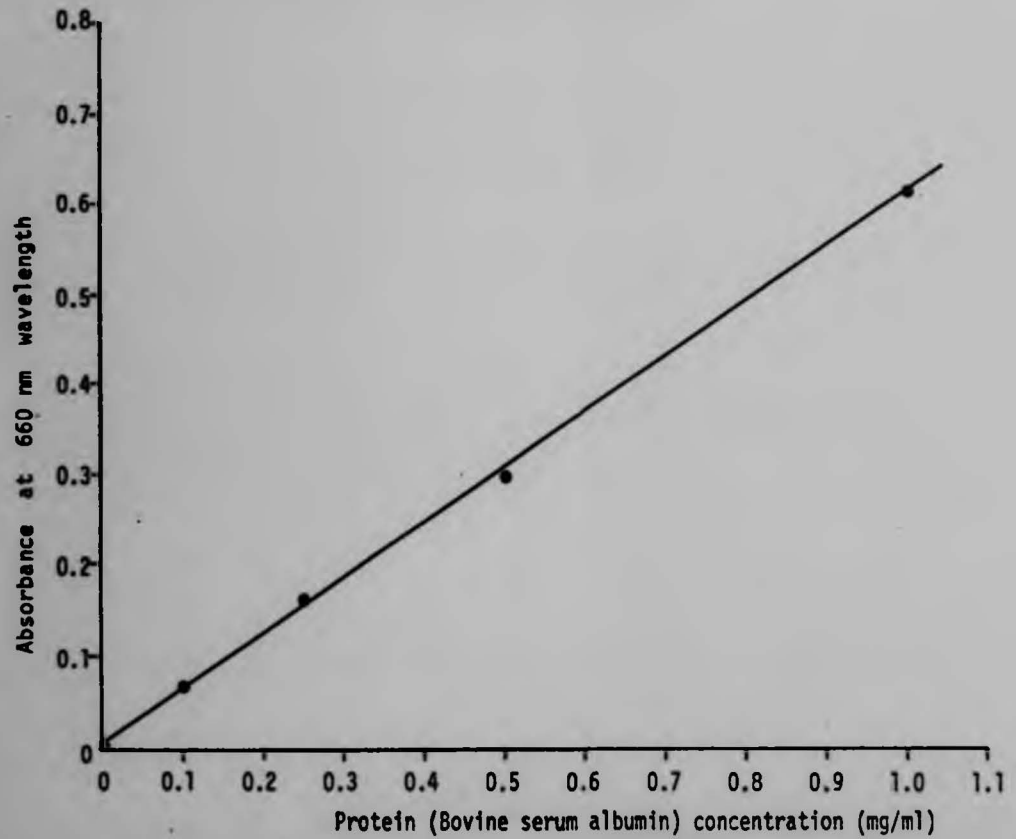


Fig. 4
Diagram of gel spreader and slot comb templates

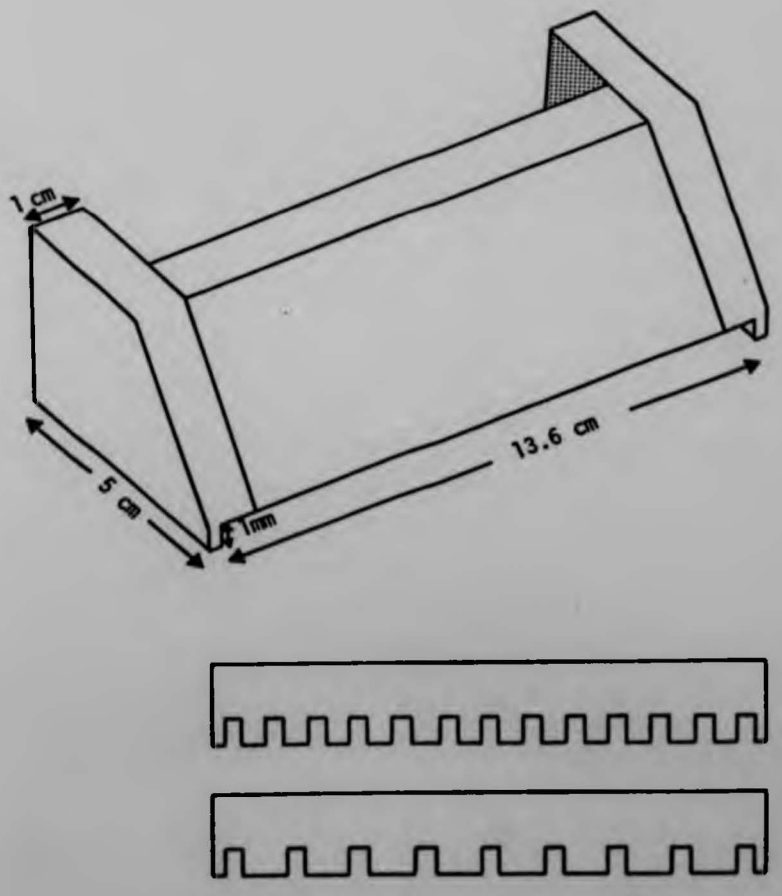


Fig. 5
Diagram of gel - forming plate

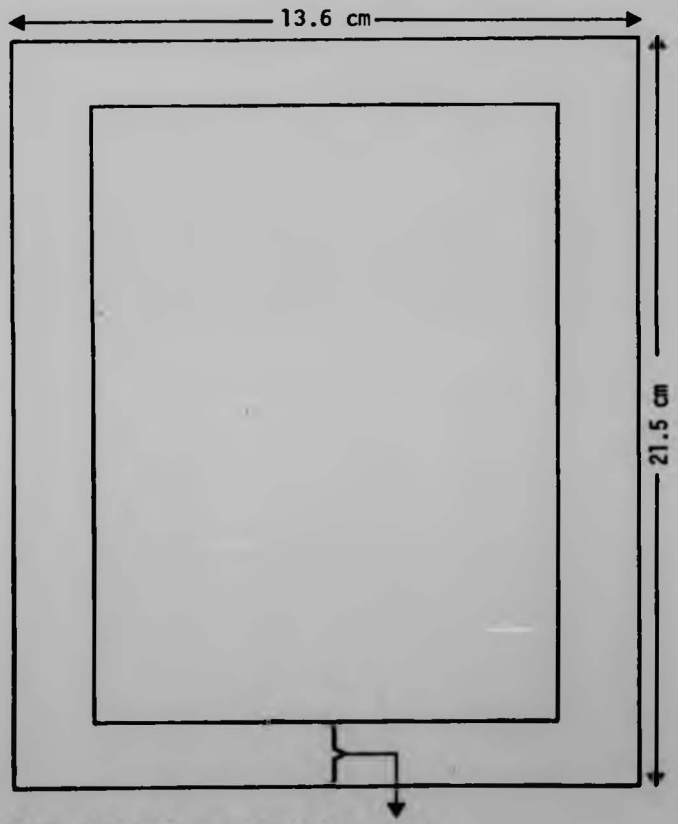
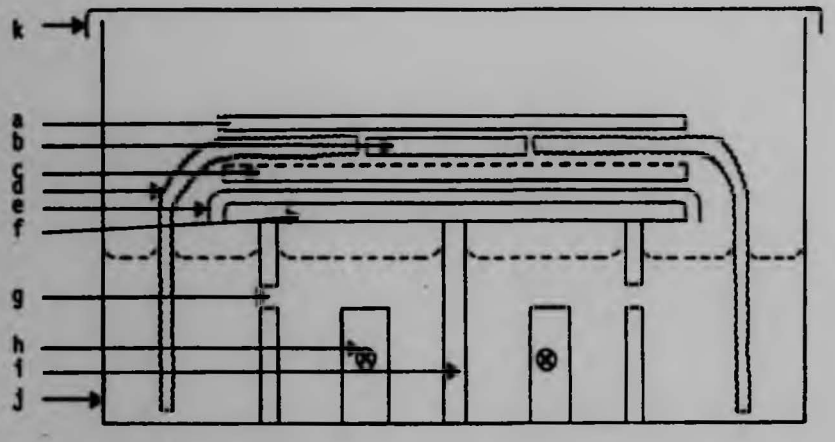


Fig. 6

Diagram showing arrangment of equipment for electrophoresis



a - Thick glass placed lengthwise
b - Thin glass plate placed widthwise
c - Starch - gel plate
d - Spontex wick
e - Melinex insulating sheet
f - Cooling plate
g - Inter connecting hole between wick and electrode chambers
h - Leads connecting to power pack
i - Ridge seperating anodic and cathodic compartments of the tank
j - Tank containing buffer
k - Tank cover

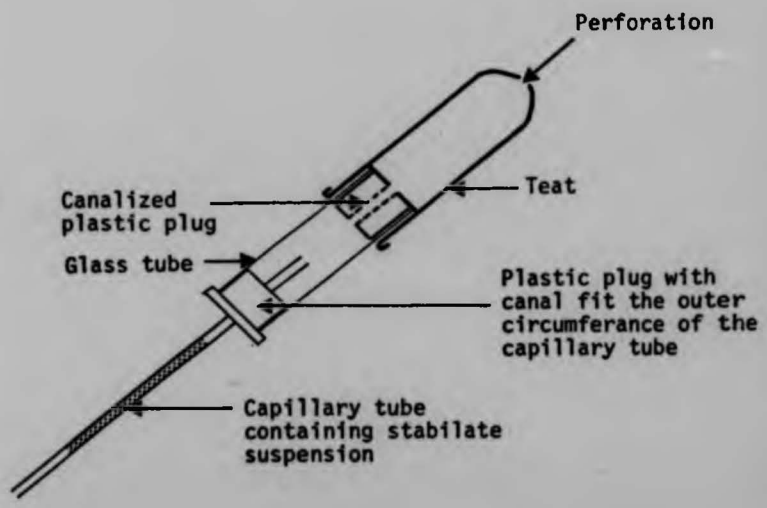


Fig. 7

"Holey - blower". For expelling stabilate suspensions from capillary tube.

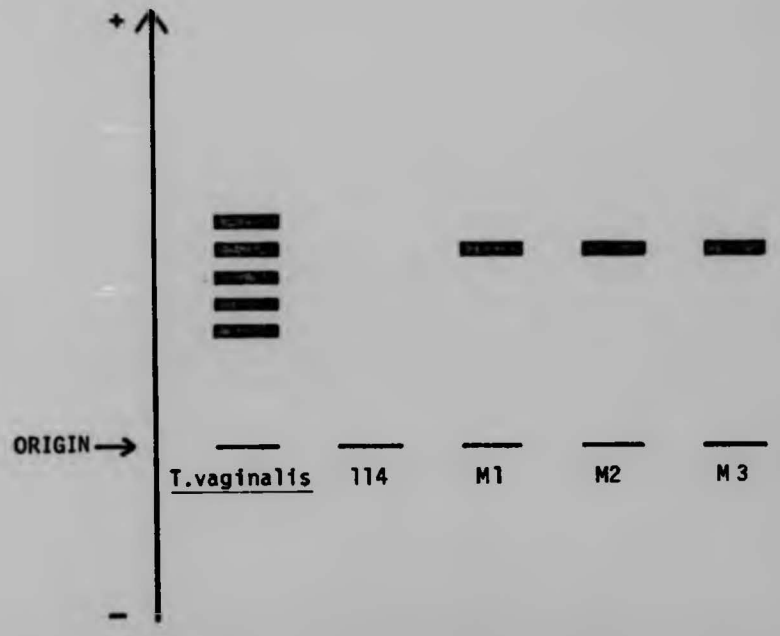


Fig. 8

Diagram summerizing electrophoretic patterns for the enzyme aldolase (ALD) of various T.vaginalis isolates (including Beckenham parent strain and mouse - virulent strain), Beckenham strain (114) and flagellates from faeces of monkey (M1&M2&M3). Dark bands represent prominent isoenzyme bands.

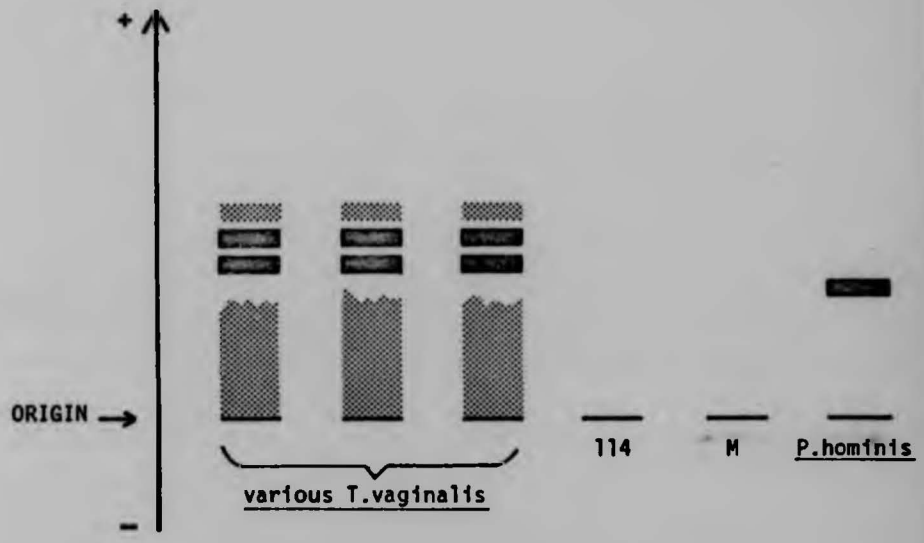


Fig. 9

Diagram summerizing electrophoretic pattern for the enzyme ME (malic enzyme "Oxaloacetate decarboxylating") of various T.vaginalis isolates (including Beckenham parent strain and mouse - virulent strain), Beckenham (hamster) strain (114), flagellates from faeces of monkey (M), and P.hominis. The pattern of this enzyme was reproducible, diffuse and streaked from the origin in T.vaginalis. Dark bands represent prominent isoenzyme bands while the lighter (stippled) bands represent weaker bands.

Fig. 10

Diagram summerizing electrophoretic patterns for the enzyme lactate dehydrogenase (LDH) of various T.vaginalis isolates (including Beckenham parent strain and mouse - virulent strain), Beckenham (hamester) strain (114) and flagellates isolated from faeces of monkey (M1 & M2 & M3). Dark bands represent prominent isoenzyme bands. Numbers (I,II,III & IV) refer to isoenzyme patterns.

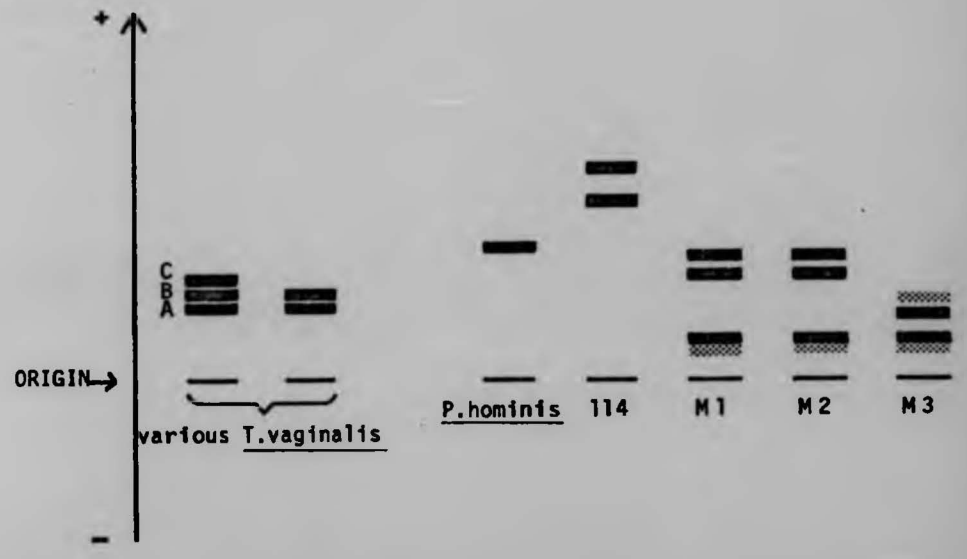


Fig. 11

Diagram summerizing electrophoretic patterns for enzyme glucose phosphate isomerase (GPI) of various T.vaginalis isolates (including Beckenham parent strain and mouse - virulent strain), P.hominis , Beckenham (hamester) strain (114) and flagellates from faeces of monkey (M1 & M2 & M3). Dark bands represent prominent isoenzyme bands; lighter (stippled) bands represent weaker isoenzyme bands.

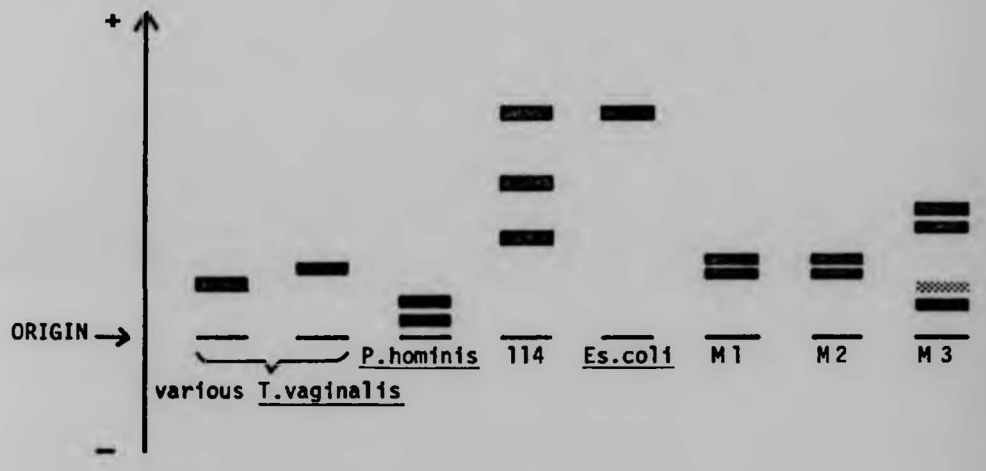


Fig. 12

Diagram summerizing electrophoretic patterns for the enzyme phasphoglucomutase (PGM), of various T.vaginalis, Pentatrichomonas hominis, Beckenham (ham ster) strain (114), Escherichia coli, and flagellates from faeces of monkey (M1 & M2 & M3). Dark bands represent prominent isoenzyme bands; lighter (stippled) bands represent weaker bands.

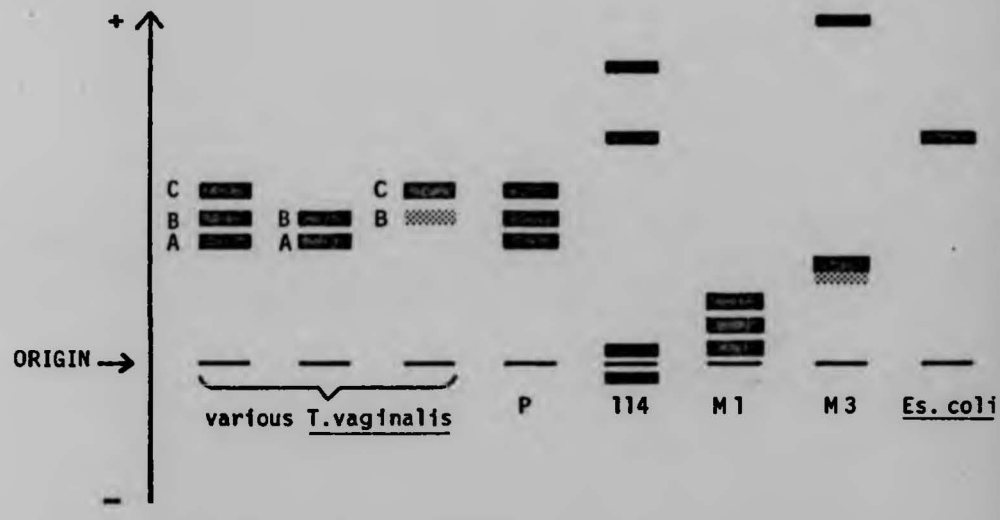


Fig. 13

Diagram summerizing electrophoretic patterns for the enzyme malate dehydrogenase (NAD^+ oxireductase)(MDH) of various T.vaginalis isolates, Beckenham (hamster) strain (114), flagellates from faeces of monkey (M1 & M3), and Escherichia coli. Dark bands represent prominent isoenzyme bands; lighter (stippled) bands represent weaker isoenzyme bands. P. represents Beckenham parent strain.

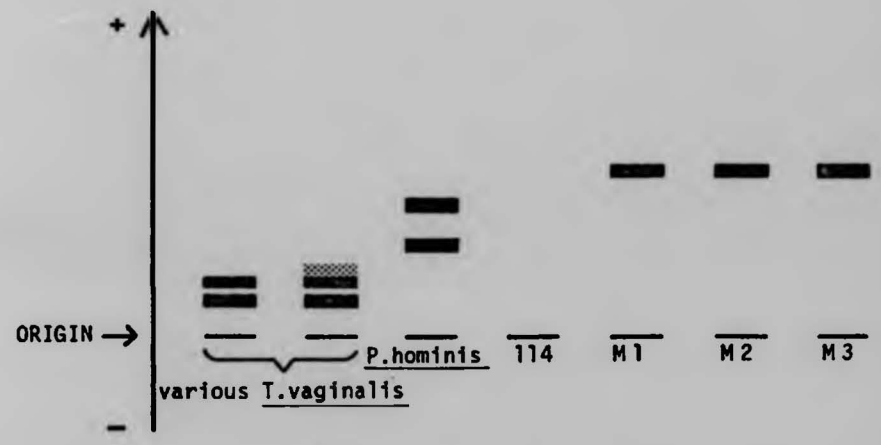


Fig. 14

Diagram summerizing electrophoretic patterns for the enzyme hexokinase (HK) of various T.vaginalis isolates including Beckenham parent strain mouse - virulent strain), Pentatrichomonas hominis, Beckenham (hamster) strain (114) and flagellates from faeces of monkey (M1 & M2 & M3). Dark bands represent prominent isoenzyme bands; lighter (stippled) band represent weaker isoenzyme band.

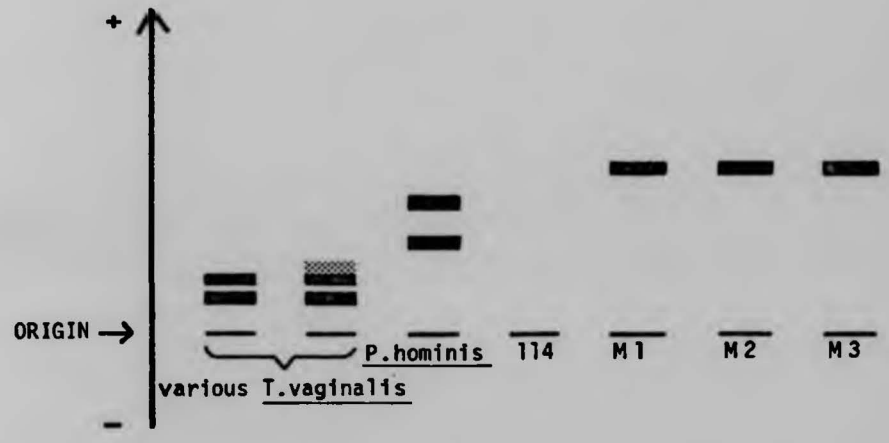


Fig. 14

Diagram summerizing electrophoretic patterns for the enzyme hexokinase (HK) of various T.vaginalis isolates including Beckenham parent strain mouse - virulent strain), Pentatrichomonas hominis, Beckenham (hamster) strain (114) and flagellates from faeces of monkey (M1 & M2 & M3). Dark bands represent prominent isoenzyme bands; lighter (stippled) band represent weaker isoenzyme band.

FIG. 15: ENZYME ASSAY CURVES

CURVES TO SHOW HOW TIME AND ABSORBANCE UNITS ARE READ OFF THE CURVES

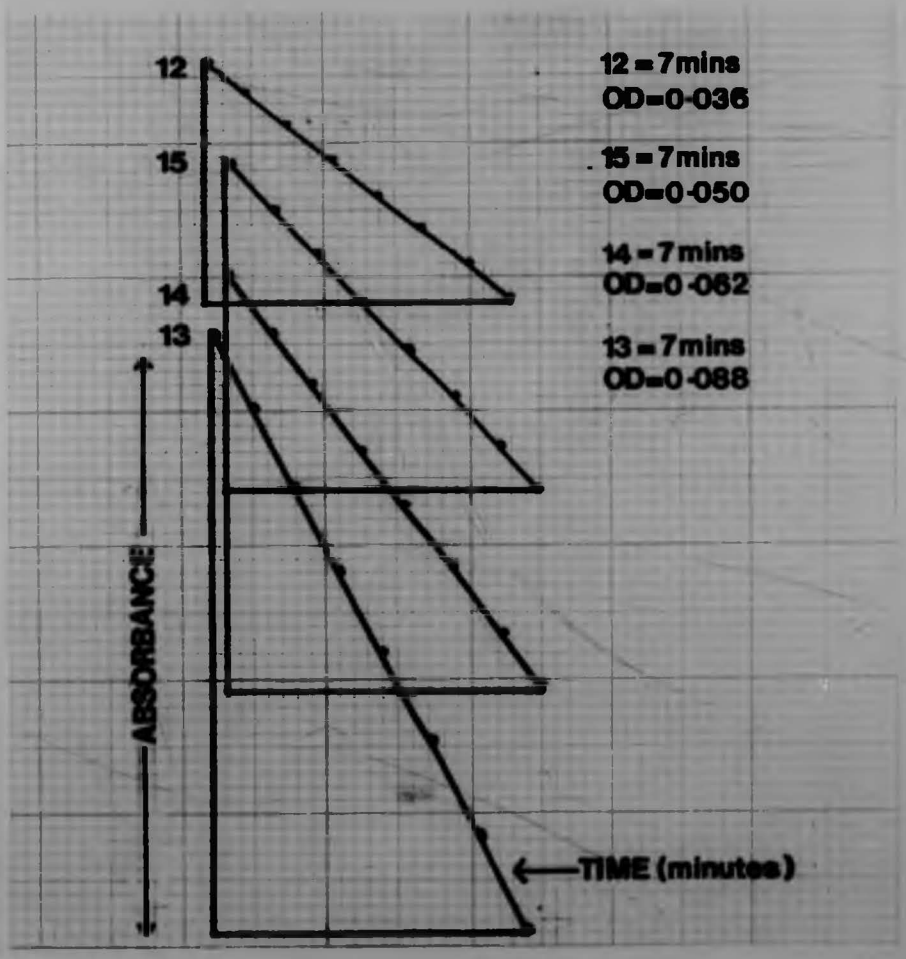


Fig. 16

Summary of different enzyme patterns of T.vaginalis

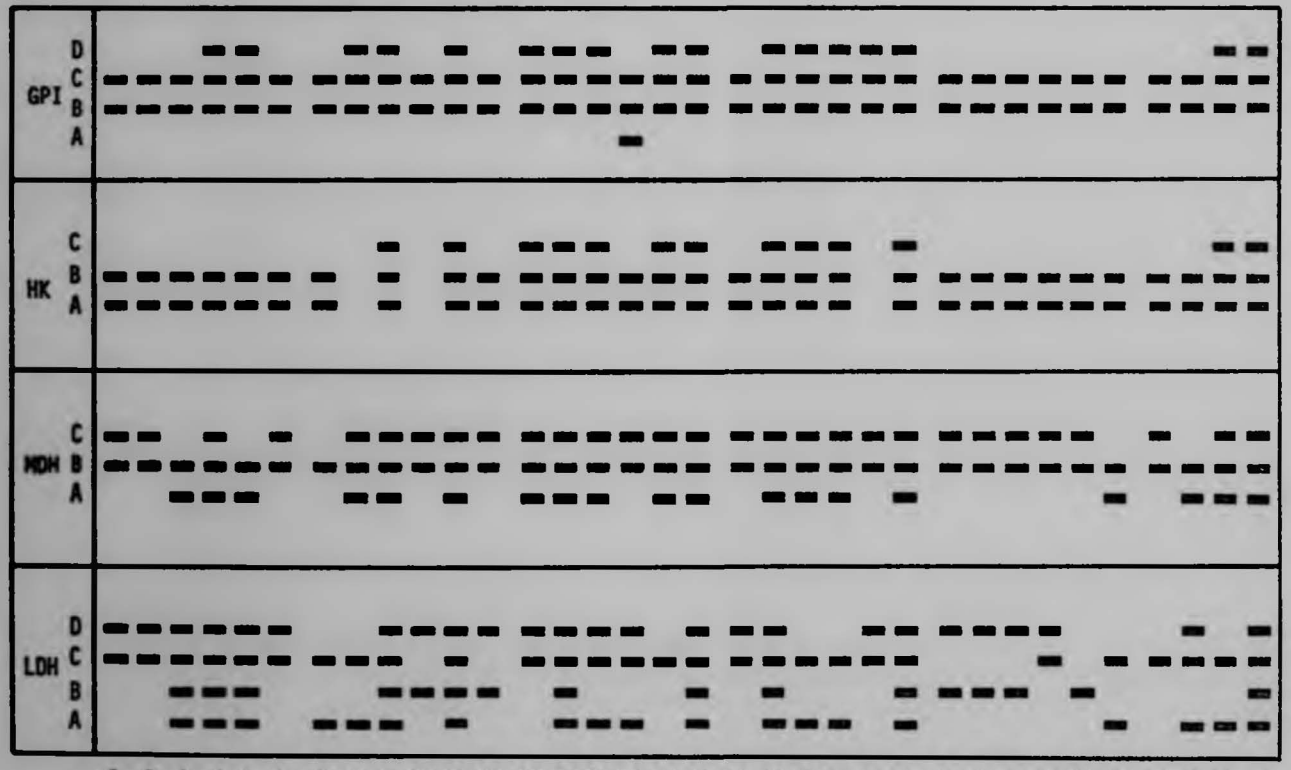


Fig. 17
Scoring of enzyme patterns of T.vaginalis

SPECIMEN NUMBER	CODE NUMBER	ENZYME PATTERNS										
		LDH				MDH			HK		GPI	
		D C B A	D B	D C	C A	C B A	B A	C B	C B A	B A	C B A	B A
76/714	TV 1			●				●		●		●
Lump 994	TV 2			●				●		●		●
Lump 996	TV 3	●					●			●		●
75/7479	TV 4	●				●			●		●	
75/7940	TV 5	●		●			●			●	●	
75/8213	TV 6				●			●		●		●
76/754	TV 7				●		●			●		●
75/8403	TV 8					●			●		●	
Lump 1314	TV 9	●				●			●		●	
Lump 1315	TV 10		●					●		●		●
75/8555	TV 11	●				●			●		●	
76/991	TV 12		●					●		●		●
76/2002	TV 13	●				●			●		●	
76/415	TV 14	●				●			●		●	
76/7689	TV 15	●				●			●		●	
Lump 1242	TV 16											
75/2040	TV 17				●	●			●		●	
75/8095	TV 18	●				●			●		●	
Lump 1160	TV 19			●				●		●		●
Lump 1165	TV 20	●				●			●		●	
Lump 1064	TV 21				●	●			●		●	
Lump 1065	TV 22				●	●			●		●	
Lump 1192	TV 23											
Lump 1193	TV 24	●				●			●		●	
76/434	TV 25		●					●		●		●
Lump 1042	TV 26		●					●		●		●
Lump 1043	TV 27		●					●		●		●
Lump 1044	TV 28			●				●		●		●
Lump 1031	TV 29		●					●		●		●
Lump 1046	TV 30				●	●	●			●		●
Lump 1264	TV 31			●				●		●		●
Lump 1232	TV 32				●		●			●		●
75/8043	TV 33				●	●			●		●	
75/8040	TV 34	●				●			●		●	

Fig. 18
Summary of grouping of T.vaginalis according to enzyme patterns

SPECIMEN NUMBER	CODE NUMBER	ENZYME PATTERNS										
		LDH				MDH			HK		GPI	
		D C B A	D B	D C	C A	C B A	B A	C B	C B A	B A	C B A	B A
75/7479	4	•				•			•		•	
Lump 1314	9	•				•			•		•	
75/8555	11	•				•			•		•	
76/415	14	•				•			•		•	
76/7689	15	•				•			•		•	
75/8095	18	•				•			•		•	
Lump 1165	20	•				•			•		•	
Lump 1193	24	•				•			•		•	
75/8040	34	•				•			•		•	
76/2002	13	•				•			•		•	
Lump 1315	10		•					•		•		•
76/991	12		•					•		•		•
76/434	25		•					•		•		•
Lump 1042	26		•					•		•		•
Lump 1043	27		•					•		•		•
Lump 1031	29		•					•		•		•
76/714	1			•				•		•		•
Lump 994	2			•				•		•		•
75/8213	6			•				•		•		•
Lump 1160	19			•				•		•		•
Lump 1044	28			•				•		•		•
Lump 1264	31			•				•		•		•
75/2040	17				•	•			•		•	
Lump 1064	21				•	•			•		•	
Lump 1065	22				•	•			•		•	
75/8043	33				•	•			•		•	
75/8403	8				•	•			•		•	
76/754	7				•		•			•		•
Lump 1046	30				•		•			•		•
Lump 1232	32				•		•			•		•
Lump 996	3	•					•			•		•

— Samples left for further investigation and not grouped in the present study are - 1192 (TV23) : because of PGM pattern
TV16 : because of GPI pattern

— Few samples were recorded directly from the plate before diffusion

Fig. 18
Summary of grouping of T.vaginalis according to enzyme patterns

SPECIMEN NUMBER	CODE NUMBER	ENZYME PATTERNS										
		LDH				MDH			HK		GPI	
		D C B A	D B	D C	C A	C B A	B A	C B	C B A	B A	C B A	B A
75/7479	4	•				•			•		•	
Lump 1314	9	•				•			•		•	
75/8555	11	•				•			•		•	
76/415	14	•				•			•		•	
76/7689	15	•				•			•		•	
75/8095	18	•				•			•		•	
Lump 1165	20	•				•			•		•	
Lump 1193	24	•				•			•		•	
75/8040	34	•				•			•		•	
76/2002	13	•				•			•		•	
Lump 1315	10		•					•		•		•
76/991	12		•					•		•		•
76/434	25		•					•		•		•
Lump 1042	26		•					•		•		•
Lump 1043	27		•					•		•		•
Lump 1031	29		•					•		•		•
76/714	1			•				•		•		•
Lump 994	2			•				•		•		•
75/8213	6			•				•		•		•
Lump 1160	19			•				•		•		•
Lump 1044	28			•				•		•		•
Lump 1264	31			•				•		•		•
75/2040	17				•	•			•		•	
Lump 1064	21				•	•			•		•	
Lump 1065	22				•	•			•		•	
75/8043	33				•	•			•		•	
75/8403	8				•	•			•		•	
76/754	7				•		•			•		•
Lump 1046	30				•		•			•		•
Lump 1232	32				•		•			•		•
Lump 996	3	•					•			•		•

— Samples left for further investigation and not grouped in the present study are = 1192 (TV23) : because of PGM pattern
TV16 : because of GPI pattern

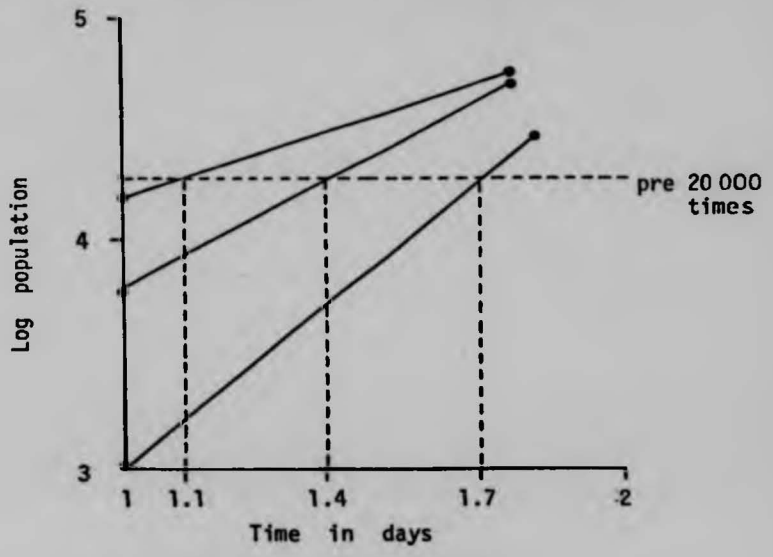
— Few samples were recorded directly from the plate before diffusion

Fig. 19
Summary of grouping of T.vaginalis according to enzyme patterns

GROUP	SPECIMEN NUMBER	CODE NUMBER	ENZYME PATTERNS				REMARKS	
			LDH	MDH	HK	GPI	Final conc of Con A (ug/ml)	Generation Time (hours)
I	75/7479	TV 4						
	Lump 1314	TV 9	D					
	75/8555	TV 11	C	C	C	C		
	76/415	TV 14	B	B	B	B	12.5 µg/ml	Long GT (slowgrow) > 11hrs
	76/7689	TV 15	A	A	A	A		
	75/8095	TV 18						
	Lump 1165	TV 20						
	Lump 1193	TV 24						
	75/8040	TV 34						
	76/2002	TV 13				with late,developed in GPI		band c
II	Lump 1315	TV 10	D					
	76/991	TV 12		C				
	76/434	TV 25	B	B	B	B		
	Lump 1042	TV 26			A	A	25 µg/ml	Medium > 6hrs and
	Lump 1043	TV 27						
	Lump 1031	TV 29						
III Similar to group II but different in LDH pattern	76/714	TV 1	D					
	Lump 994	TV 2	C	C				<11hrs
	75/8213	TV 6		B	B	B		
	Lump 1160	TV 19			A	A		
	Lump 1044	TV 28					50 µg/ml	
	Lump 1264	TV 31						
IV Similar to group I but different in LDH pattern	75/2040	TV 17						
	Lump 1064	TV 21	C	C	C	C		
	Lump 1065	TV 22		B	B	B		
	75/8043	TV 33	A	A	A	A		
	75/8403	TV 8						
V	76/754	TV 7	C				100 µg/ml	Short GT (fast grow) < 6hrs
	Lump 1046	TV 30						
	Lump 1232	TV 32		B	B	B		
	Lump 996	TV 3	A	A	A	A		

Fig. 20a

Graphical method for estimation of pre 20 000 time* for T.vaginalis



a = inoculum of 400 T.vaginalis
b = inoculum of 200 T.vaginalis
c = inoculum of 100 T.vaginalis

* Pre 20 000 time is defined as the time taken for a given inoculum to grow up to 20 000 [illegible]/ml.

Fig. 20b

The relationship between pre 20 000 times and log inoculum of culture

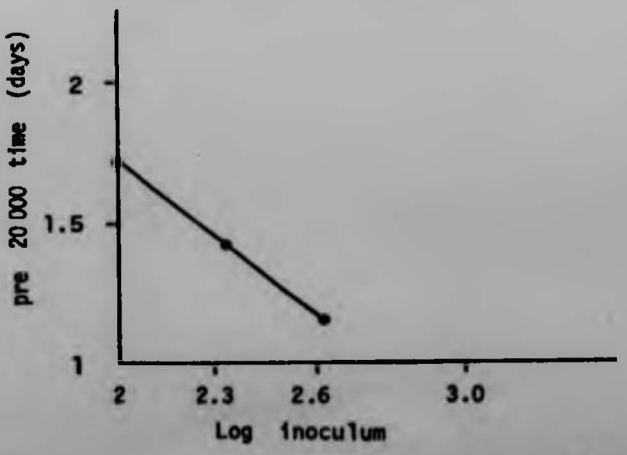


Figure 21: Photograph showing (from left to right) upper surface of Hemming's filter (A), lower surface of Hemming's filter with projecting rim (B), assembled Hemming's filter (C), bijou bottle with sponge at top socket of Hemming's filter (D), and a bijou bottle with sponge used in collection of vaginal secretion.

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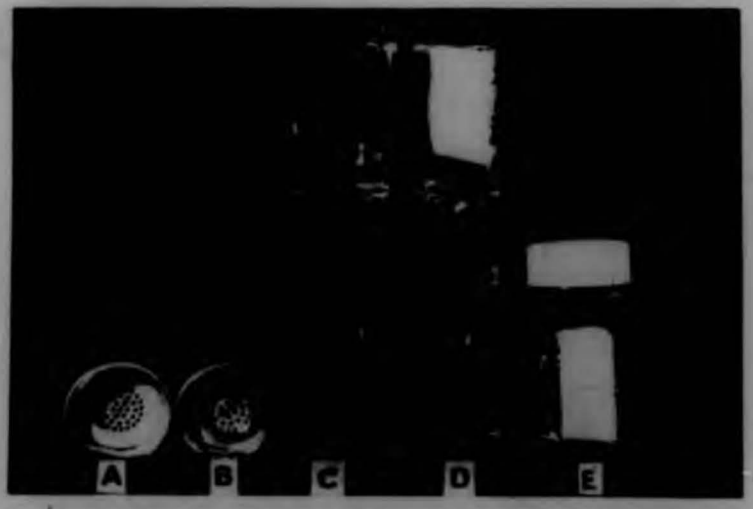
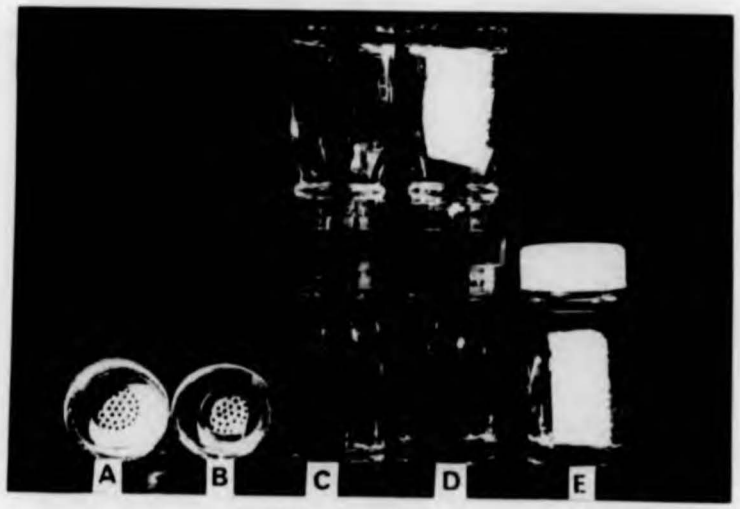
21



to right)
ter (A),
ter with
Hemming's
sponge at
(D), and
d in col-

21



Figures 22, 23, 24: Photomicrograph (x 1000) of non-dividing T. vaginalis. Different shape (contour) can be seen in preparation stained with Giemsa.

Key: AF: Four anterior flagella
UMR: Undulant membrane and recurrent flagellum
B: Blepharoplast
N: Nucleus
A: Axostyle

22

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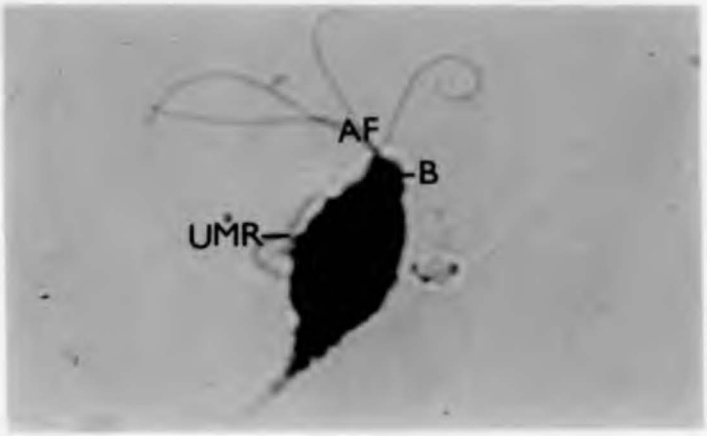
24

22



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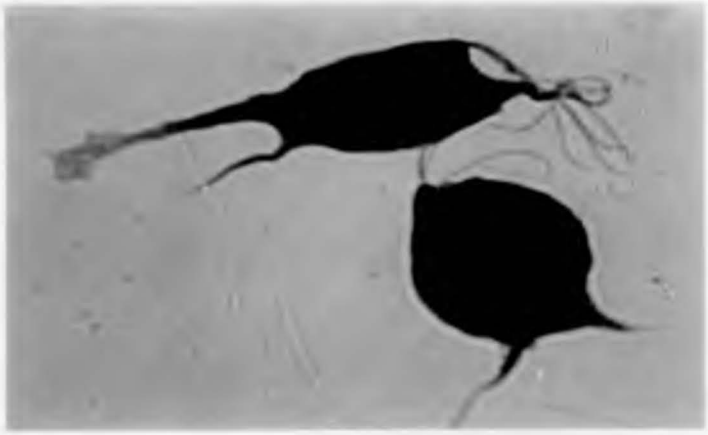

24

Figure 25: Photomicrograph (x 1000) of dividing *T. vaginalis* with four nuclei, two at each pole. Preparation stained with Giemsa.

Figure 26: Photomicrograph (x 1000) of dividing *T. vaginalis* with one nucleus at each pole. Preparation stained with Giemsa.

Figure 27: Photomicrograph (x 1000) of dividing *T. vaginalis* with two nuclei at the anterior end. Preparation stained with Giemsa.

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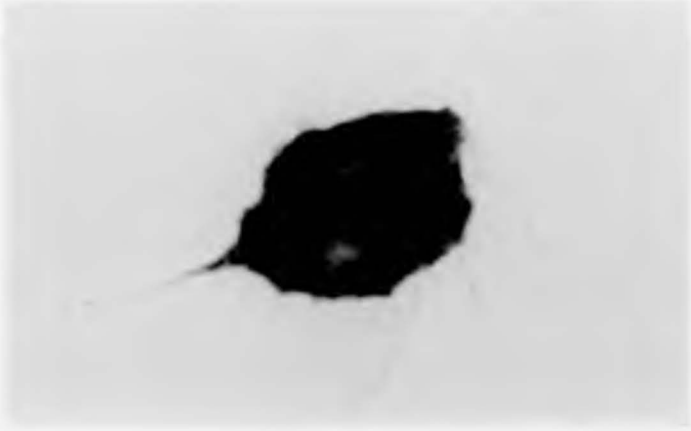

Figure 28: Photomicrograph (x 1000) of abnormal form (giant form) of T. vaginalis, grown in MLM. Stained with Giemsa.

Figure 29: Photomicrograph (x 1000) of dividing abnormal form (giant form) of T. vaginalis with four nuclei. Stained with Giemsa.

Figure 30: Photomicrograph (x 1000) of dividing abnormal form (giant form) of T. vaginalis with two nuclei. Stained with Giemsa.

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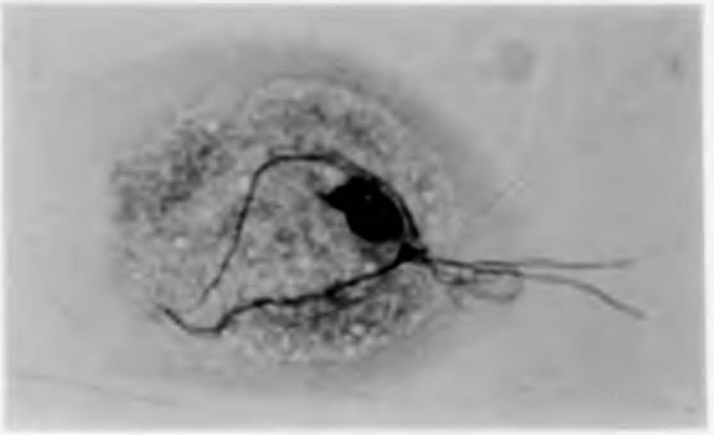

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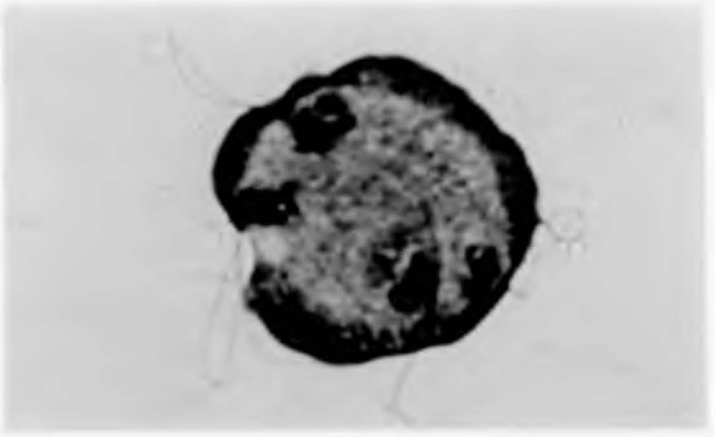

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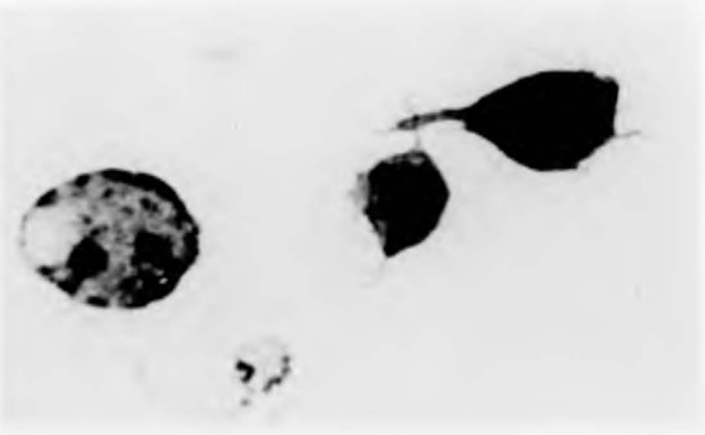

Figure 31: Photomicrograph (phase-contrast; x 1000) of flagellate isolated from faeces of monkey with engulfed starch granules.

Figure 32: Photomigrograph (x 1000) of flagellate isolated from faeces of monkey stained with Giemsa. Note the three anterior flagella and the undulating membrane which extends posteriorly.

Figures 33, 34: Photomicrograph (x 1000) of flagellate isolated from faeces of monkey stained with Giemsa showing undulating membrane and starch granules.

31

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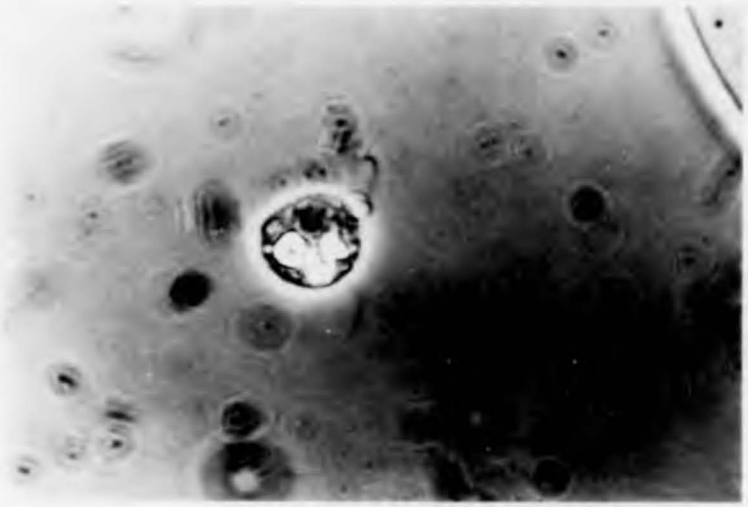

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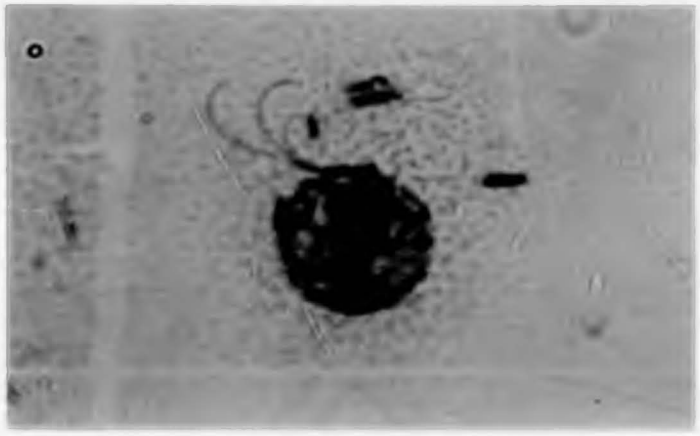
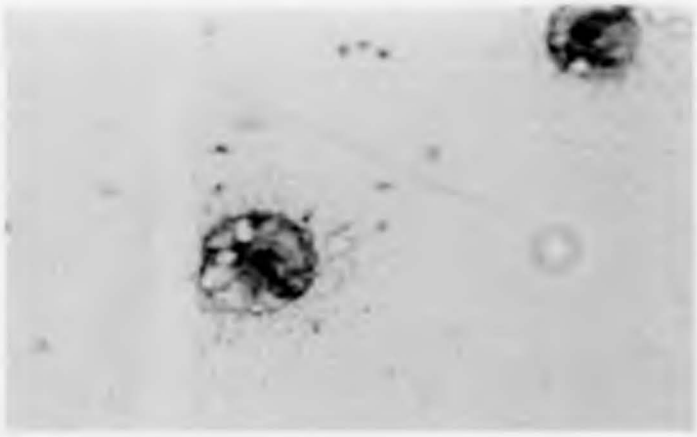


33



Figures 35, 36: Photomicrograph (x 1000) of Beckenham (hamster) strain grown in Stenton's medium and stained with Giemsa. Note the trailing recurrent flagellum and the undulating membrane which extends beyond the anterior half of the body.

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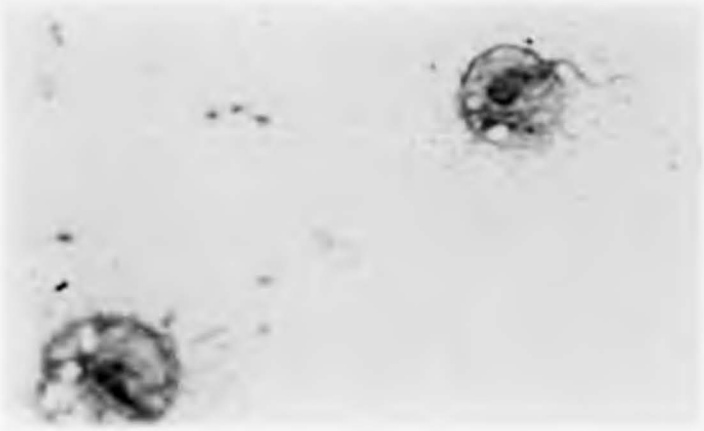
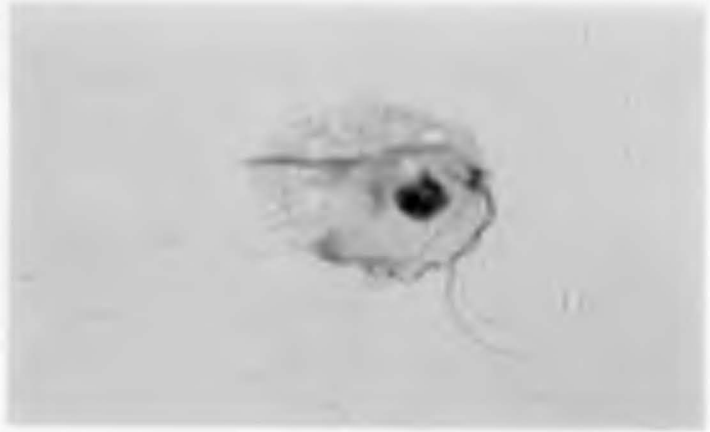
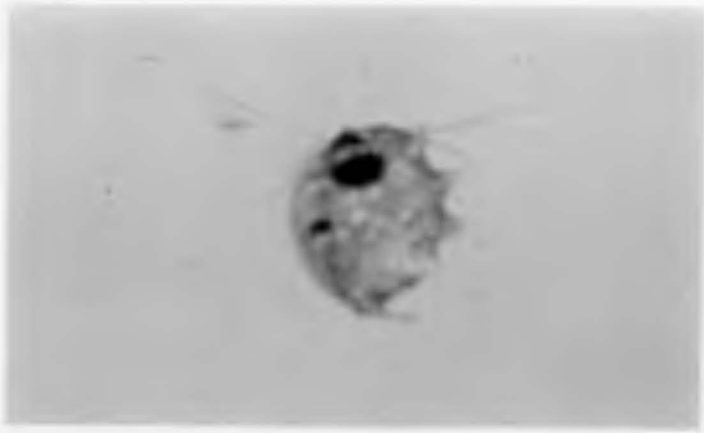


35



36



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Figures 37, 38, 39, 40: Photomicrograph (x 1000) of Beckenham (hamster) strain (from hamster vagina) stained with Giemsa to show the trailing recurrent flagellum and undulating membrane extending beyond the anterior half of the body.

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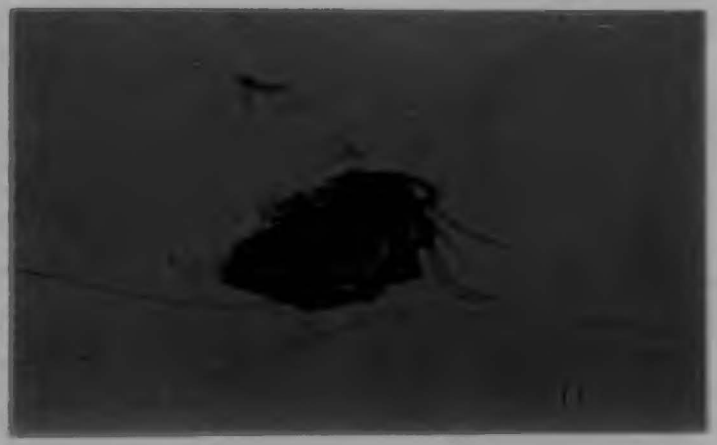
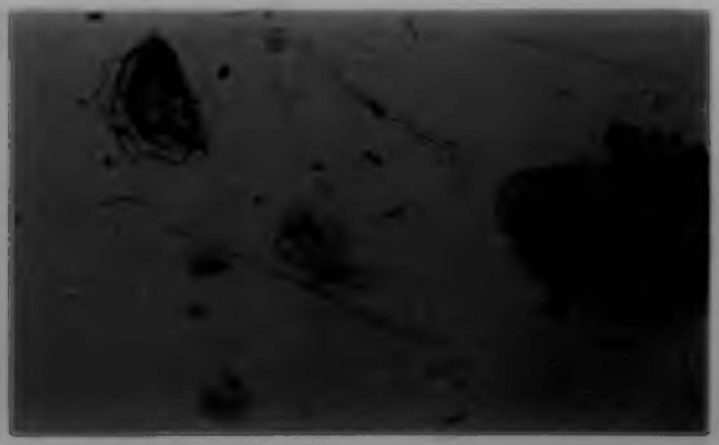


38



x 1000)
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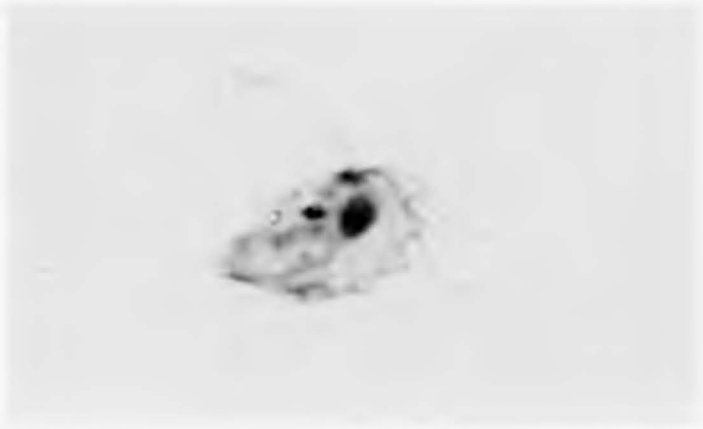

38

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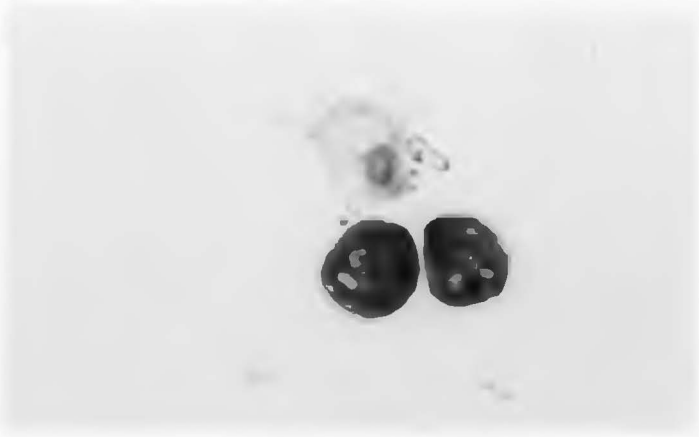


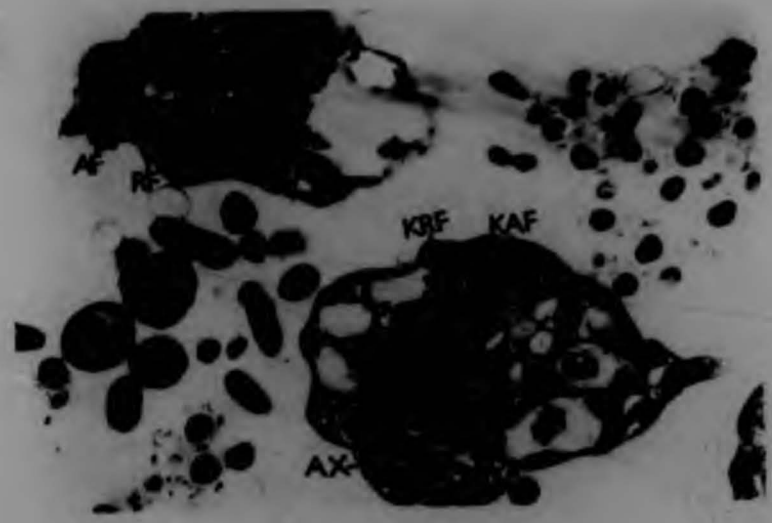
Figure 41: An electron micrograph of thin section of flagellate isolated from faeces of monkey (x 12,500).

Key:
- C: Costa
- AF: Three anterior flagella
- RF: Recurrent flagellum
- N: Nucleus
- KAF: Kinetosome of the anterior flagella
- KRF: Kinetosomes of the recurrent flagellum
- Ax: Axostyle

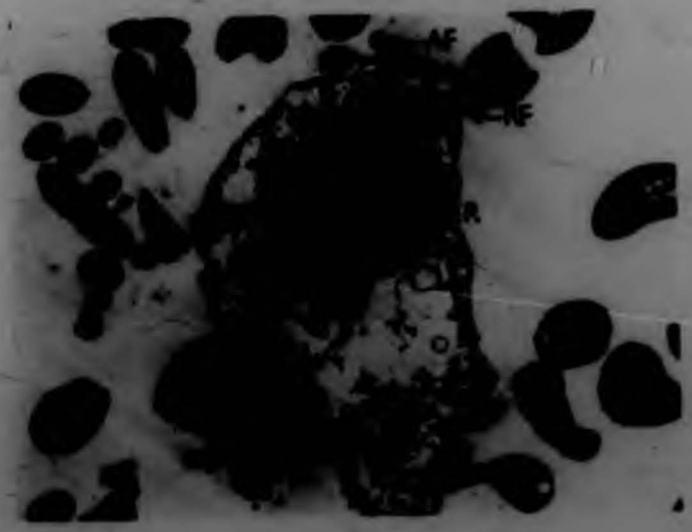
Figure 42: An electron micrograph of thin section of the flagellate isolated from faeces of monkey (x 12,500).

Key:
- ER: Endoplasmic reticulum
- N: Nucleus
- AF: Three anterior flagella
- RF: Recurrent flagellum
- Ax: Axostyle
- H: Granules (probably paraxostylar hydrogenosomes)

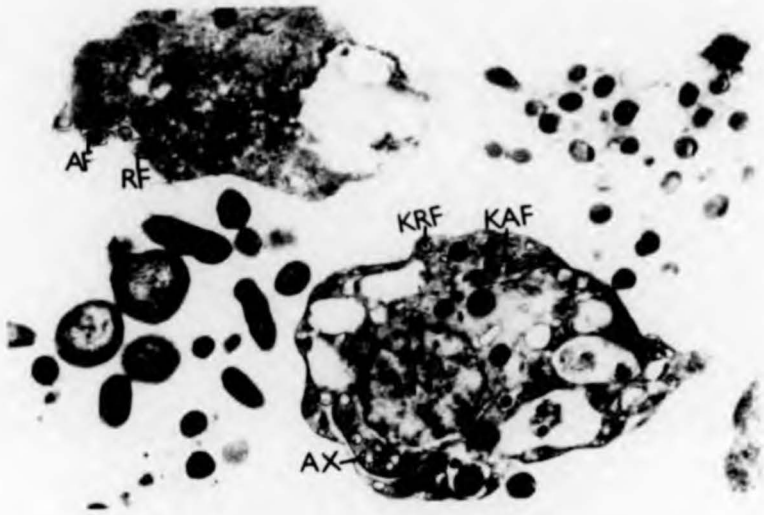
41



42



41



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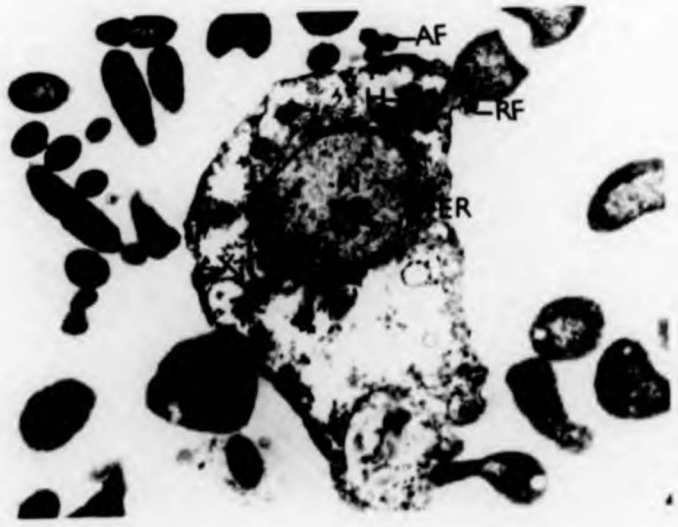

Figure 43: Higher magnification of an electron micrograph of thin section of the flagellate isolated from faeces of monkey (x 35,000).

Key: FV: Food vacuole
AF: Three anterior flagella
RF: Recurrent flagellum
CE: Cytoplasmic extension) undulating
ML: Marginal lamella) membrane
GG: Glycogen granules

Figure 44: Higher magnification of an electron micrograph of thin section of the flagellate isolated from faeces of monkey (x 80,000). Cross-section of the undulating membrane, typical of these organelles in Trichomonadinae. The membrane of the recurrent flagellum (MRF) appears in this section not to be connected to the fin-like dorsal cytoplasmic extension (CE).

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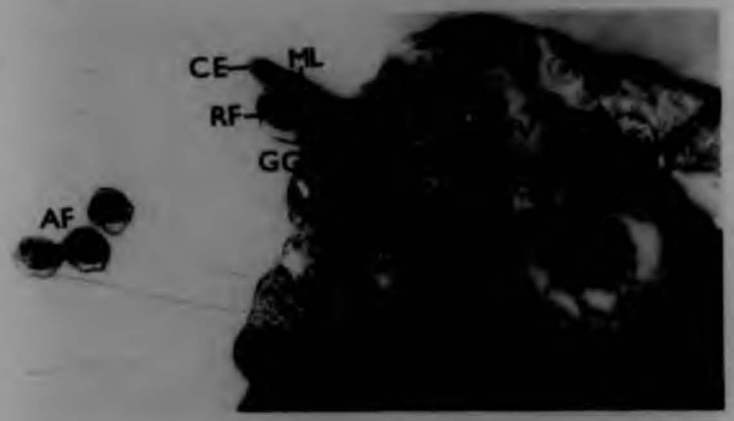

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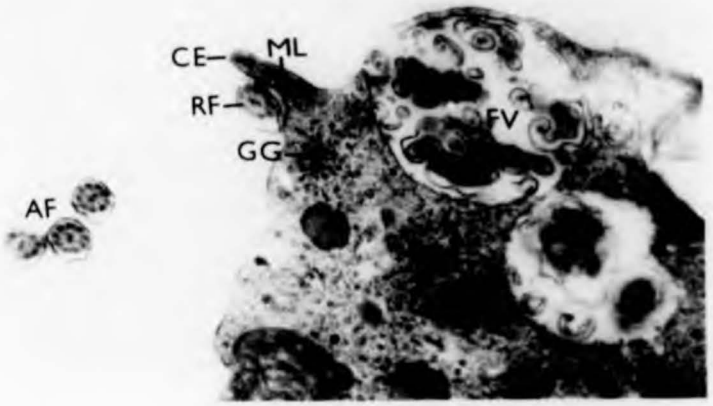

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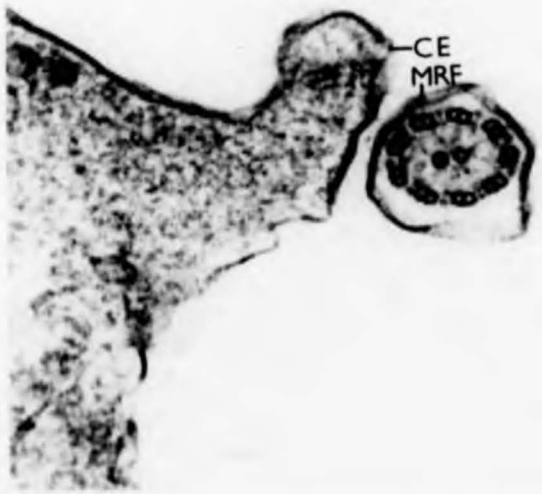

Figure 45: An electron micrograph of the anterior flagella of flagellate isolated from faeces of monkey. The anterior flagella, in cross section, are seen in the periflagellar canal (PC) (x 125,000).

Figure 46: An electron micrograph of thin section of the flagellate isolated from faeces of monkey (x 8,000).

Key: SG: Starch granule engulfed by the parasite
N: Nucleus
C: Costa
AF: Anterior flagella (three) enclosed in periflagellar canal
RF: Recurrent flagellum
PF: Parabasal filament

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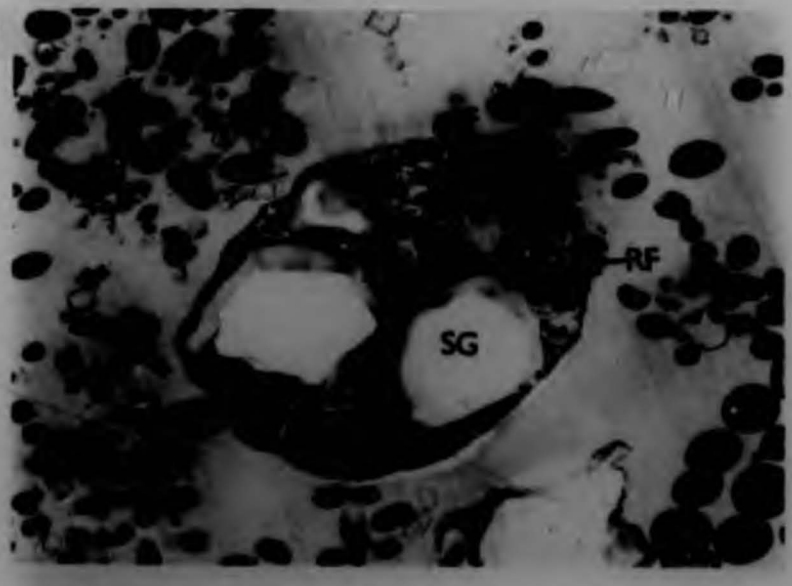

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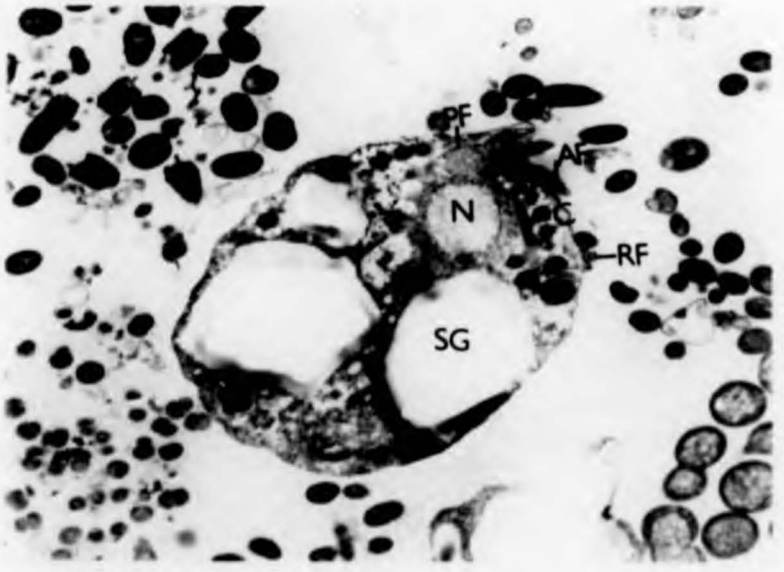

Figure 47: An electron micrograph of cross section of flagellate isolated from faeces of monkey showing costa (C), parabasal fibre (PF) and paracostal granules (PCG; probably most of them are hydrogenosomes) (x 80,000).

Figure 48: An electron micrograph showing longitudinal section of the costa (C) with Type A periodicity (Honigberg 1978a) shown in flagellate isolated from faeces of monkey. The pattern of such costa consisting of multilinear repeating units. A row of paracostal granules (PCG; probably many of them are hydrogenosomes) parallels the costa (x 80,000).

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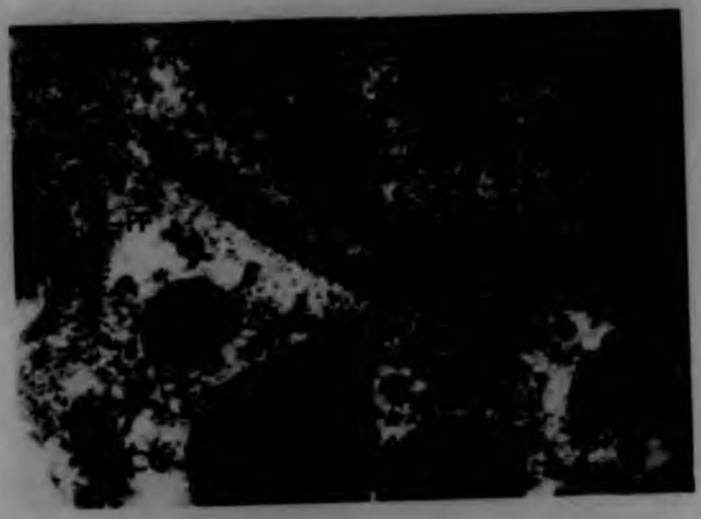

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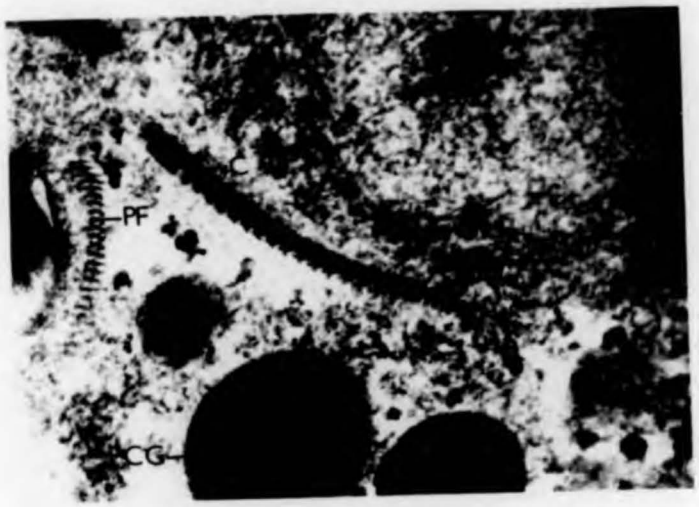

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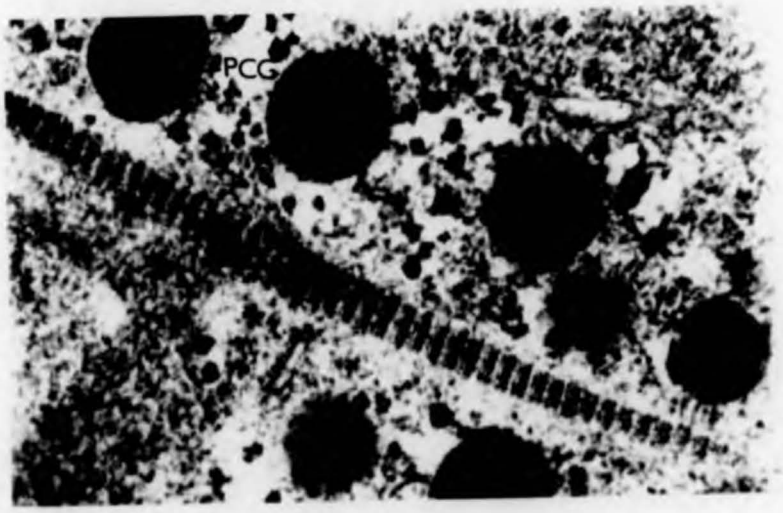

Figure 49: An electron micrograph of flagellate isolated from faeces of monkey (x 80,000).

Key: PCG: Paracostal granules (probably many of them are hydrogenosomes)

KAF: Kinetosome of anterior flagellum

T: Rootlet filament

PF: Parabasal fibre

C: Costa

Figure 50: An electron micrograph of flagellate isolated from faeces of monkey (x 120,000).

Key: KAF: Kinetosomes of anterior flagella

KRF: Kinetosome of recurrent flagellum

PF: Parabasal filaments

T: Rootlet filament

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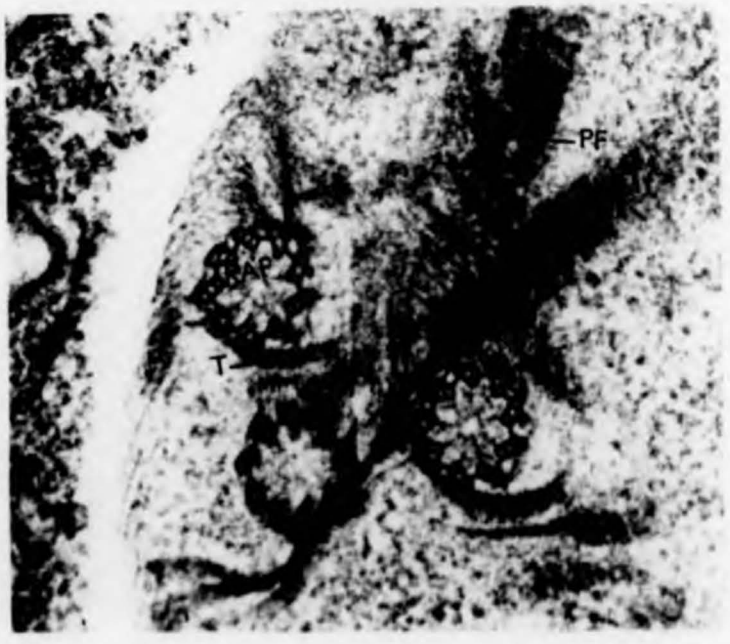

Figure 51: Colonies of T. vaginalis in agar plate inoculated with 0.1 ml of suspension containing approximately 1000 organisms per ml^{-1}.

Figure 52: Colonies of T. vaginalis in agar plate inoculated with 0.1 ml of suspension containing approximately 10,000 organisms ml^{-1}.

Figure 53: Colonies of T. vaginalis in agar plate inoculated with 0.1 ml of suspension containing approximately 100,000 organisms ml^{-1}. The colonies are confluent and look as if photograph is out of focus, but the petridish margin shows that the focus is sharp.

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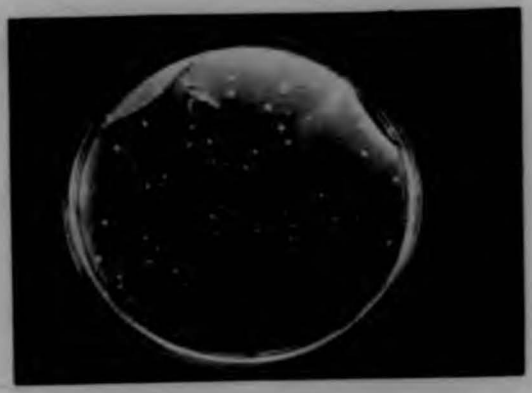

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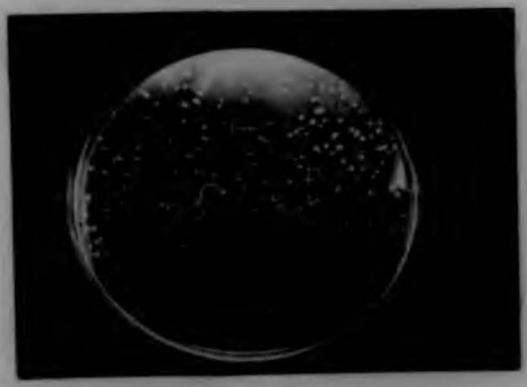

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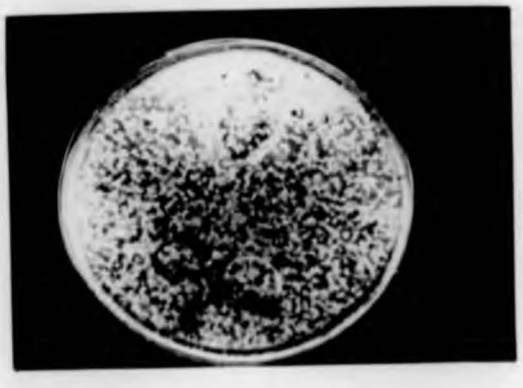

Figure 54: Photograph of an electrophoretic plate for the enzyme aldolase (ALD) of different isolates of T. vaginalis grown in modified Lumsden medium. Note the same pattern among the different isolates.

Figure 55: Photograph of an electrophoretic plate for the enzyme aldolase (ALD) of different isolates of T. vaginalis grown in modified Lumsden medium. Each isolate shows the same pattern. Note bowing which occurs with high voltage.

Figure 56: Photograph of an electrophoretic plate for the enzyme aldolase (ALD) of different isolates of T. vaginalis grown in modified Lumsden medium. Note the fastest band is hidden by the frame used during development.

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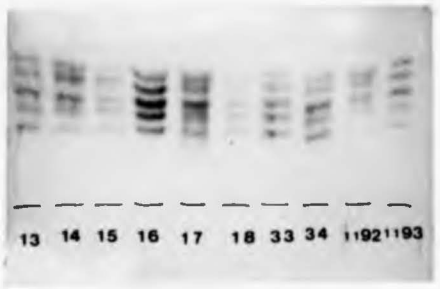
13 14 15 16 17 18 33 34 1192 1193

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18 19 20 21 22 25 26 27 28 29

Figure 57: Photograph of the same plate as that shown in Figure 56 after further incubation. Note diffusion of previous developed bands and appearance of bands in samples Nos. 25, 26, 27. Still longer incubation led to bands appearing in Samples Nos. 28 and 29.

Figure 58: Photograph of an electrophoretic plate for the enzyme aldolase (ALD) of different isolates of T. vaginalis grown in modified Lumsden medium (MLM). Note twice the usual amount of lysate has been applied with samples Nos. 26, 27, 28 and 29.

Figure 59: Photograph of the same plate as in Figure 58 after prolonged incubation.

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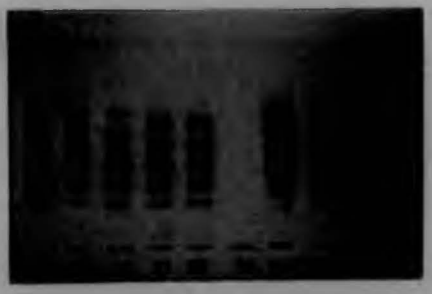

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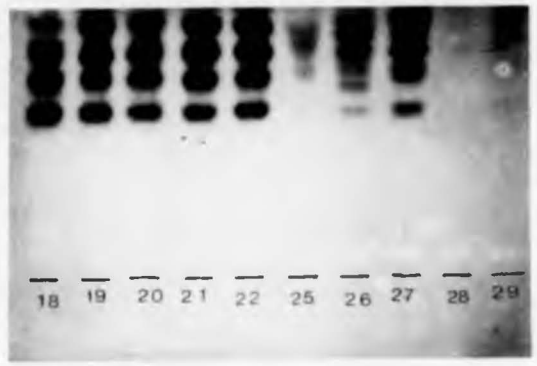
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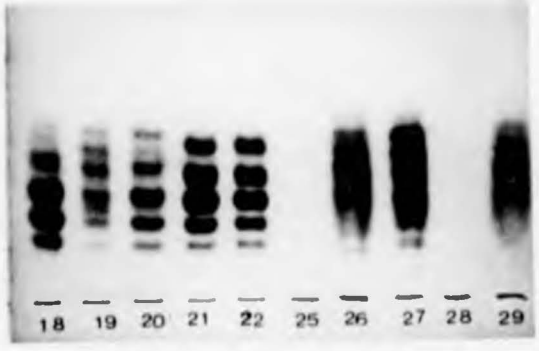
18 19 20 21 22 25 26 27 28 29

Figures 60, 61: Photograph of zymograms for malic enzyme (oxaloacetate decarboxylating) (ME) of different isolates of T. vaginalis. Each isolate shows the same pattern.

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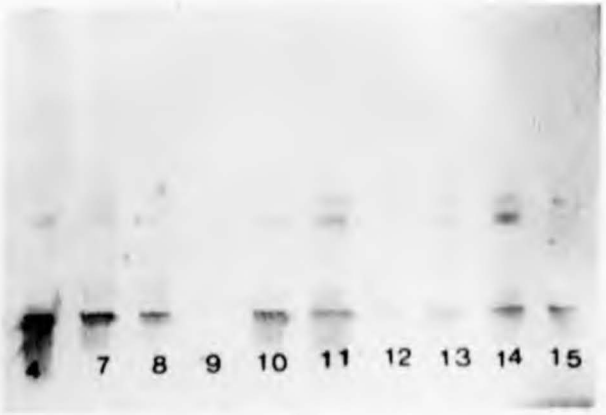

Figure 62: Photograph of an electrophoretic plate for the enzyme aldolase (ALD) of different isolates of T. vaginalis (grown in modified Lumsden medium) and monkey flagellate (M) (grown in Robinson's medium).

Figure 63: Photograph of zymogram of malic enzyme (oxaloacetate decarboxylating) (ME) of different isolates of T. vaginalis.

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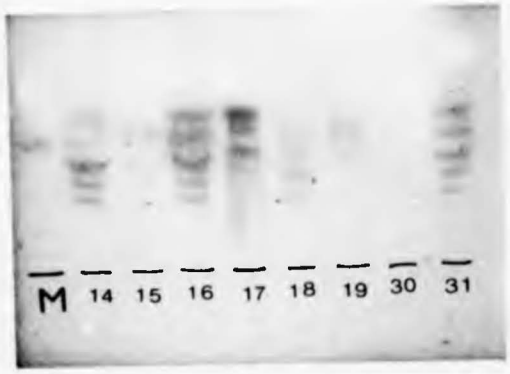

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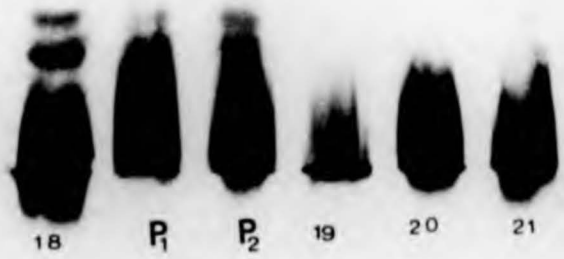

Figure 64: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of T. vaginalis using Tris/PO4 buffer (tank and gel) pH 8.1.

Figure 65: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of T. vaginalis using Tris/Maleate buffer (tank and gel) pH 7.8 (Compare Figure 64).

Figure 66: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of T. vaginalis. Tris/ Maleate buffer pH 7.8 (tank and gel) was used for this and all subsequent LDH plates.

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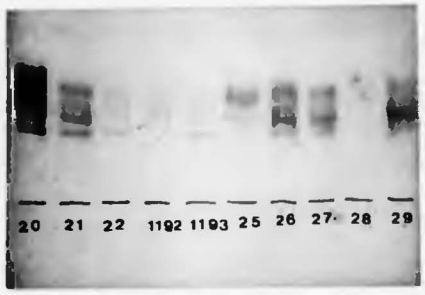

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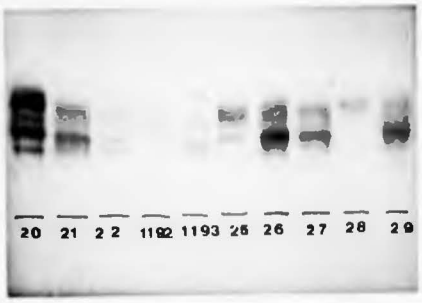

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Figure 67: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of T. vaginalis.

Figure 68: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of monkey flagellate (M) and different isolates of T. vaginalis (14, 15, 16, 17, 18, 19, 30 and 31), grown in modified Lumsden's medium.

Figures 69, 70: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of cloned T. vaginalis. Samples in Figure 70 are not cloned.

Figure 67: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of T. vaginalis.

Figure 68: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of monkey flagellate (M) and different isolates of T. vaginalis (14, 15, 16, 17, 18, 19, 30 and 31), grown in modified Lumsden's medium.

Figures 69, 70: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of cloned T. vaginalis. Samples in Figure 70 are not cloned.

Figure 67: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of T. vaginalis.

Figure 68: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of monkey flagellate (M) and different isolates of T. vaginalis (14, 15, 16, 17, 18, 19, 30 and 31), grown in modified Lumsden's medium.

Figures 69, 70: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of different isolates of cloned T. vaginalis. Samples in Figure 70 are not cloned.

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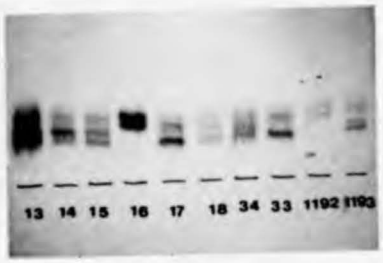
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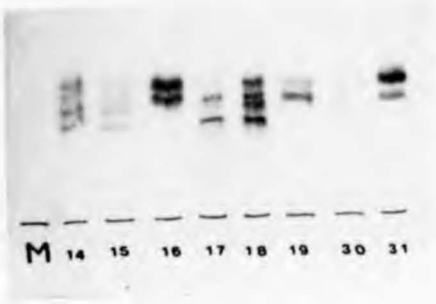
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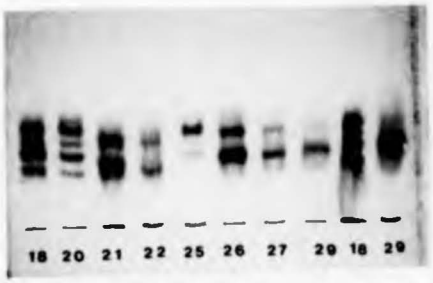
18 20 21 22 25 26 27 29 18 29

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Figure 71: Photograph of an electrophoretic plate for the enzyme lactate dehydrogenase (LDH) of Beckenham (hamster) strains (114 and 113), Beckenham parent strain grown in two different medium (modified Lumsden medium (P_1) and Stenton's medium (P_2)), different isolates of T. vaginalis (16 and 17), mixed samples (16 + 17), and monkey flagellates (M2 and M3).

Figure 72: Photograph of an electrophoretic plate for the enzyme glucose-6-phosphate dehydrogenase (G6PD) of different isolates of T. vaginalis.

Figure 73: Photograph of control plate with substrate omitted from the reaction mixture.

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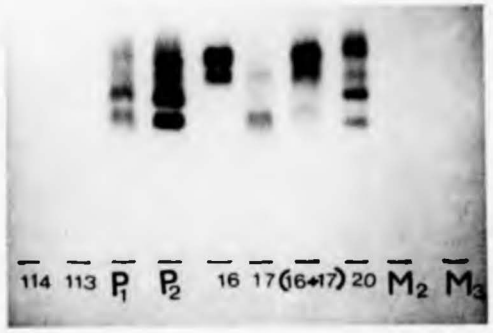

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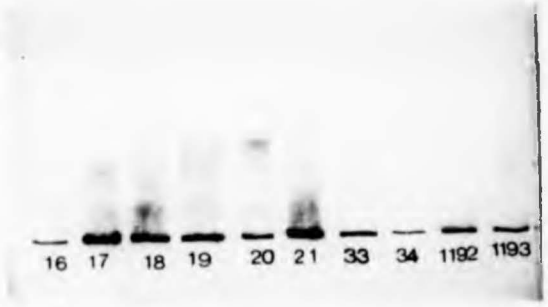

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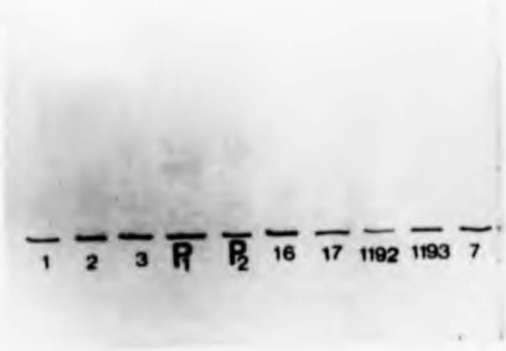

Figure 74: Photograph of an electrophoretic plate for the enzyme glucophosphate isomerase (GPI) of different isolates of T. vaginalis, mouse-virulent strains (1192 and 1193) and different isolates of flagellate from faeces of monkey (M1 and M2 and M3).

Figure 75: Photograph of an electrophoretic plate for the enzyme glucophosphate isomerase (GPI) of different isolates of T. vaginalis.

Figure 76: Photograph of an electrophoretic plate for the enzyme glucophosphate isomerase (GPI) of different isolates of T. vaginalis and Beckenham (hamster) strains (113 and 114).

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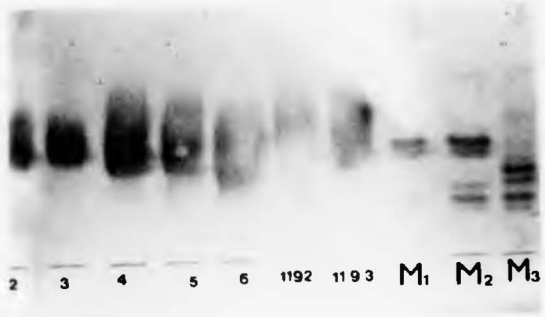
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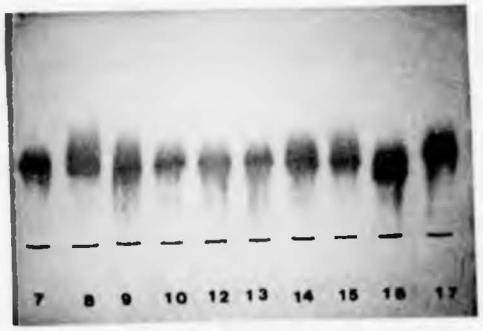
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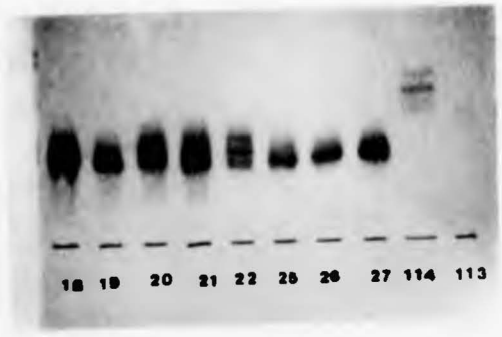
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113

Figure 77: Photograph of an electrophoretic plate for the enzyme glucose phosphate isomerase (GPI).

Key: 114 & 113: Beckenham (hamster) strains

34870 (P_1 & P_2): Beckenham parent strain grown in modified Lumsden medium (P_1) and Stenton's medium (P_2)

16,17 & 20: different isolates of T. vaginalis

16 + 17: mixed extracts of T. vaginalis (16 + 17)

M2 + M3: different isolates of monkey flagellate

Note that bands are given by 114 (see Figure 76), but are not seen inside the developing frame on this plate.

Figure 78: Photograph of an electrophoretic plate for the enzyme phosphoglucomutase (PGM) of different isolates of T. vaginalis (1, 2, 3, 5 and 6), Beckenham (hamster) strains (113 and 114), monkey flagellate (M2) and mouse-virulent strains (1192 and 1193).

Figure 79: Photograph of an electrophoretic plate for the enzyme phosphoglucomutase (PGM).

Key: 2,3,4,5,6: different isolates of T. vaginalis

1192 & 1193: mouse-virulent strains

M1, M2, M3: different isolates of monkey flagellate.

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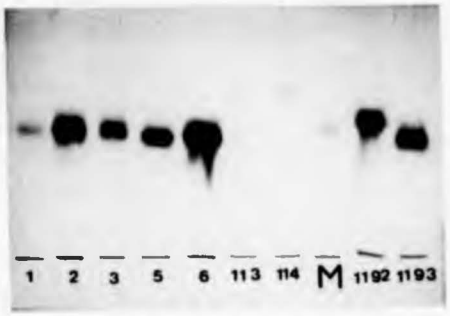

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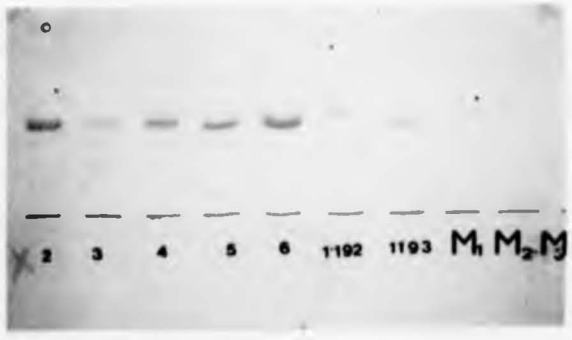

Figure 77: Photograph of an electrophoretic plate for the enzyme glucose phosphate isomerase (GPI).
Key: 114 & 113: Beckenham (hamster) strains
34870 (P_1 & P_2): Beckenham parent strain grown in modified Lumsden medium (P_1) and Stenton's medium (P_2)
16,17 & 20: different isolates of T. vaginalis
16 + 17: mixed extracts of T. vaginalis (16 + 17)
M2 + M3: different isolates of monkey flagellate
Note that bands are given by 114 (see Figure 76), but are not seen inside the developing frame on this plate.

Figure 78: Photograph of an electrophoretic plate for the enzyme phosphoglucomutase (PGM) of different isolates of T. vaginalis (1, 2, 3, 5 and 6), Beckenham (hamster) strains (113 and 114), monkey flagellate (M2) and mouse-virulent strains (1192 and 1193).

Figure 79: Photograph of an electrophoretic plate for the enzyme phosphoglucomutase (PGM).
Key: 2,3,4,5,6: different isolates of T. vaginalis
1192 & 1193: mouse-virulent strains
M1, M2, M3: different isolates of monkey flagellate.

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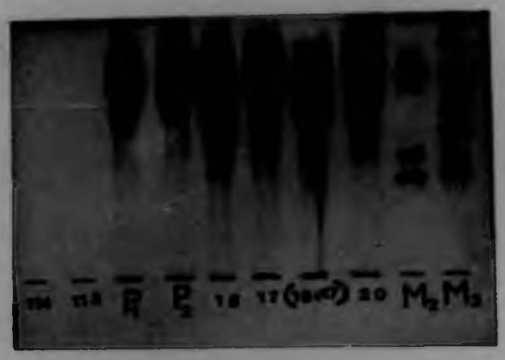

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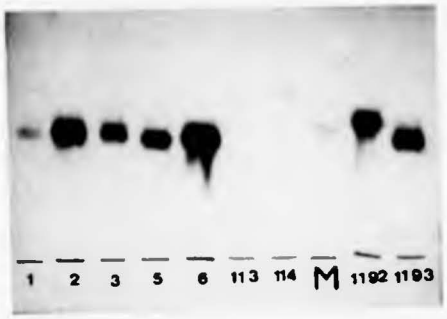
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Figure 80: Photograph of same plate as Figure 79 after prolonged incubation. Note the appearance of bands from the monkey flagellate and the faster band of 1192, also the diffusion of previously developed bands.

Figure 81: Photograph of an electrophoretic plate for the enzyme phosphoglucomutase (PGM) of different isolates of T. vaginalis. Note that each sample in this plate shows the same pattern.

Figure 82: Photograph of an electrophoretic plate for the enzyme phosphoglucomutase (PGM) of different isolates of T. vaginalis (18, 19, 20, 21, 22, 25, 26, 27) and Beckenham (hamster) strains (114,113). Beckenham (hamster) strain (114) Escherichia coli (E) and Beckenham parent strain grown in MLM (P_1) and Stenton's medium (P_2) are shown in the left side.

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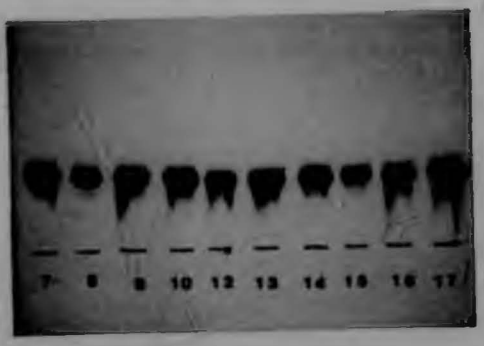

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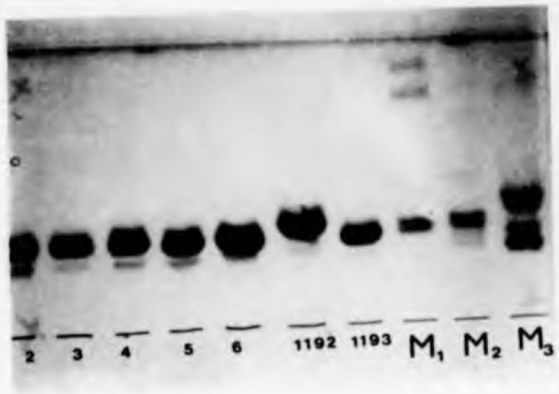

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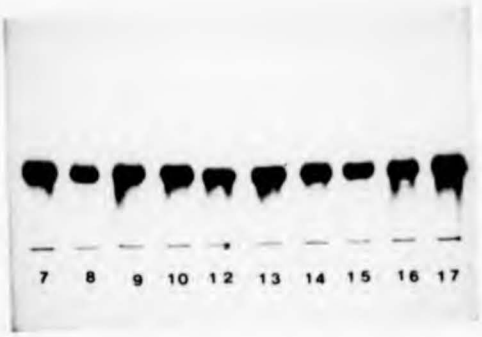

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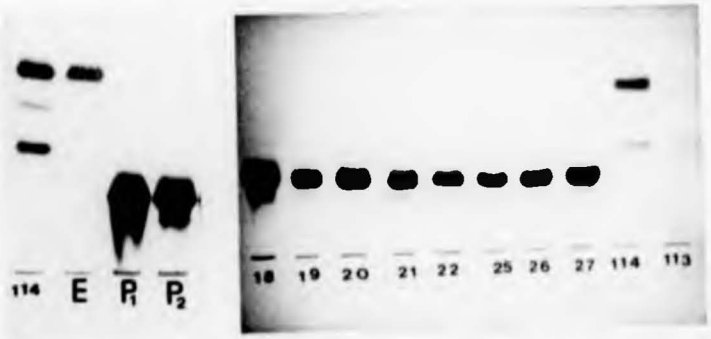

Figures 83, 84, 85: Photograph of an electrophoretic plate at different periods of incubation for the enzyme malate dehydrogenase (oxaloacetate $NADH^{+}$) (MDH) of different isolates of T. vaginalis (1, 2, 3, 5, 6), monkey flagellate (M1), Escherichia coli, Beckenham (hamster) strains (113, 114) and mouse-virulent strain (1193). On prolonged incubation, more bands appeared while the others began to diffuse.

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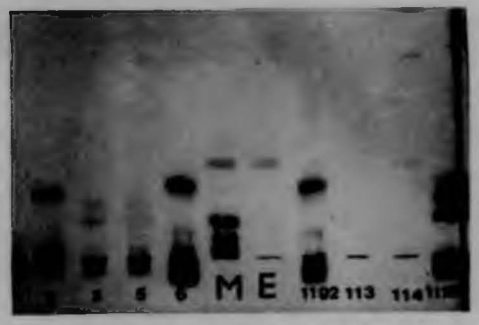

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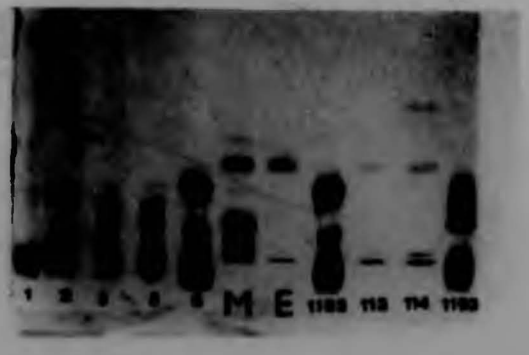

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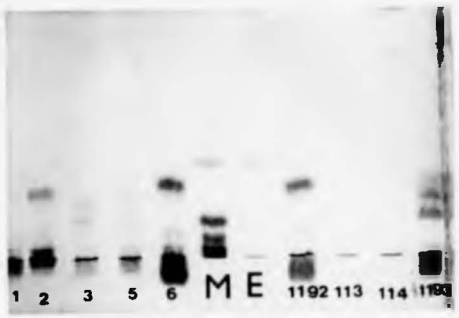

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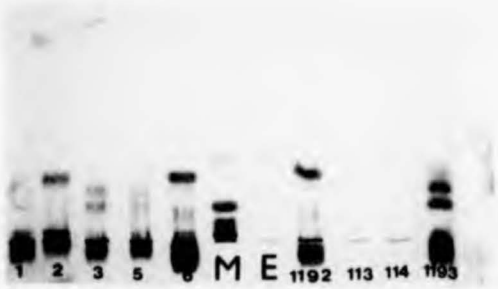

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Figure 86: Photograph of an electrophoretic plate for the enzyme malate dehydrogenase (oxireductase $NADH^+$) (MDH) of different isolates of T. vaginalis (run for two hours.)

Figure 87: Photograph of an electrophoretic plate as in Figure 86 (run for three hours).

Figure 88: Photograph of an electrophoretic plate for the enzyme malate dehydrogenase (oxireductase $NADH^+$) (MDH) of Beckenham (hamster) strains (114, 113), Beckenham parent (34870) grown in MLM (P_1) and in Stenton's medium (P_2), different isolates of T. vaginalis (16, 17), a mixture of two different isolates of T. vaginalis (16 + 17) and different isolates of monkey flagellates (M2 and M3).

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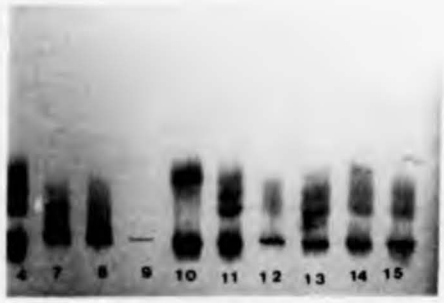

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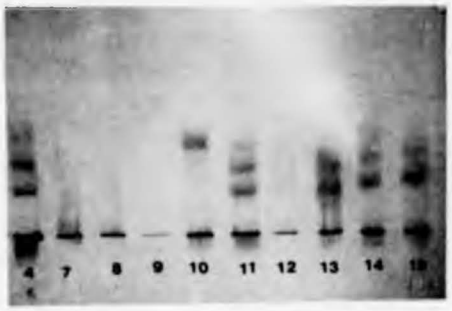

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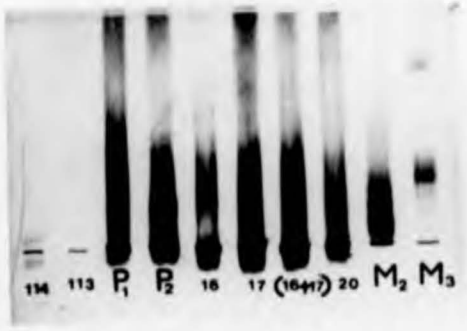

Figures 89, 90, 91: Photographs of zymogram for the enzyme malate dehydrogenase (oxireductase: $NADH^+$) (MDH) of various T. vaginalis strains. In these plates different batches of extracts for the same strains are compared. No significant differences in the pattern between different batches prepared from the same strain were ever observed.

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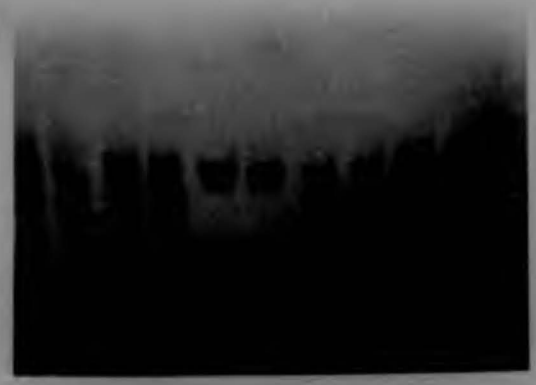

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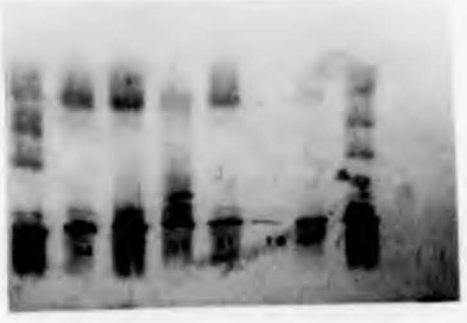

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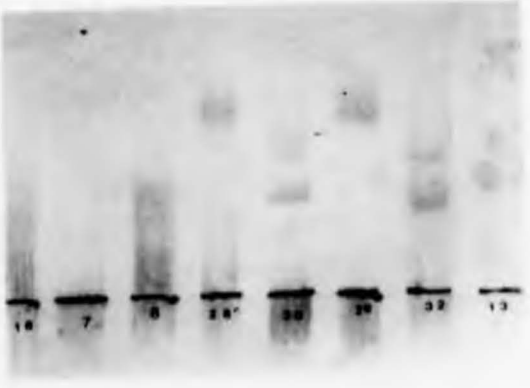

Figure 92: Photograph of an electrophoretic plate for the enzyme hexokinase (HK) of different isolates of T. vaginalis (origin of samples near to cathode).

Figure 93: Photograph of an electrophoretic plate for the enzyme hexokinase (HK) of different isolates of T. vaginalis as in Figure 92 but with origin of samples midway between cathode and anode.

Figure 94: Photograph of an electrophoretic plate for the enzyme hexokinase (HK) of different isolates of T. vaginalis (1, 2, 3, 4, 5, 6, 7) and different isolates of flagellate from faeces of monkey (M1, M2, M3).

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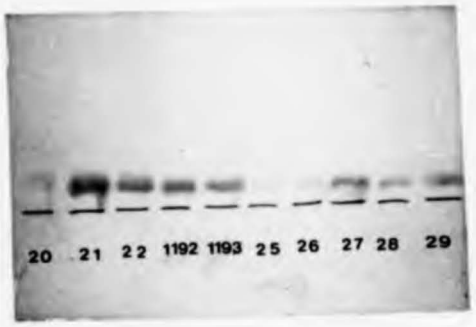

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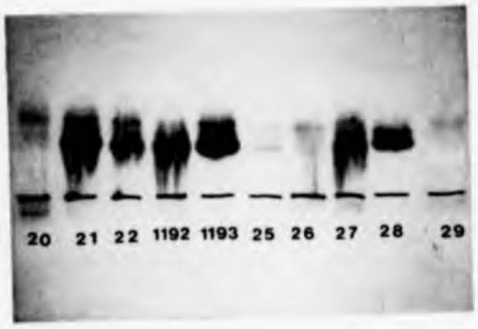

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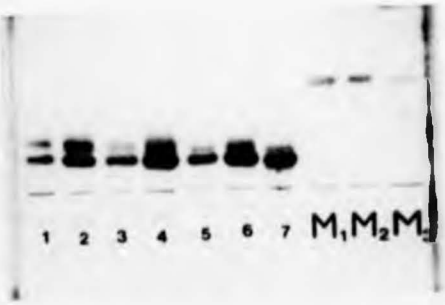

Figure 95: Photograph of same plate as in Figure 94 after 3 minutes' more incubation.

Figure 96: Photograph of an electrophoretic plate for the enzyme hexokinase (HK) of different isolates of T. vaginalis.

Figure 97: Photograph of an electrophoretic plate for the enzyme hexokinase (HK) of different isolates of T. vaginalis (first 5 slots from the left) and two different isolates of Entamoeba histolytica (last two slots to right) used as control.

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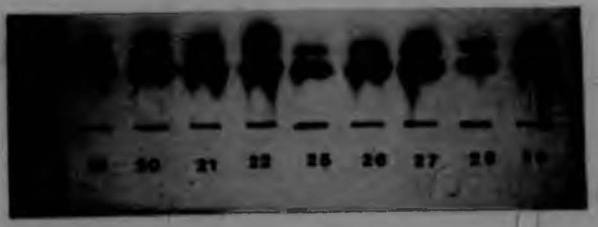

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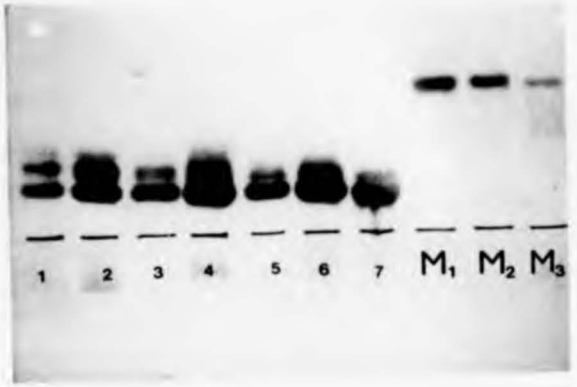

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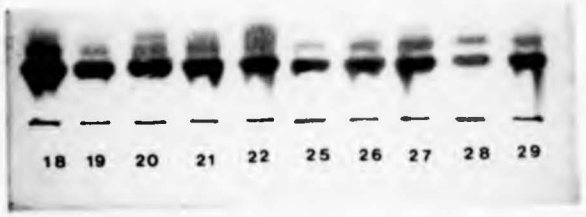

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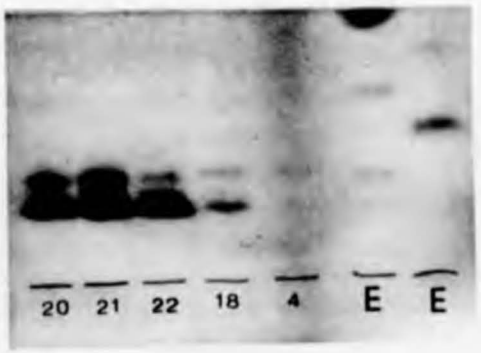

Figure 98: Photograph of an electrophoretic plate for the enzyme hexokinase (HK). Different batches of extract from the same strains of T. vaginalis are compared. No difference in the pattern could be seen between different batches prepared from the same strain.

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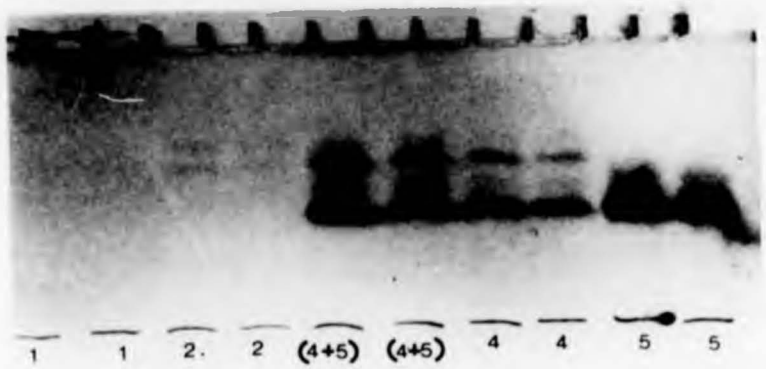

Figure 99: Photomicrograph (x 100) showing concanavalin A-induced agglutination (200 µg/ml) of T. vaginalis (1315 (Tv10)) axenically grown in modified Lumsden's medium. Note the very large clumps contain hundreds of organisms and the absence of unagglutinated T. vaginalis.

Figure 100: Photomicrograph (x 100) showing concanavalin A-induced agglutination (100 µg/ml) of T. vaginalis (1315(Tv10)). A few isolated Trichomonas, and many variable clumps (smaller than those in Figure 99) with 50 - 100 organisms/clump may be seen.

Figure 101: Photomicrograph (x 100) showing concanavalin A-induced agglutination (50 µg/ml) of T. vaginalis (1315 (Tv10)). Small clumps (20 - 50 organisms/clump interspersed with more isolated Trichomonas than in Figure 100 may be seen.

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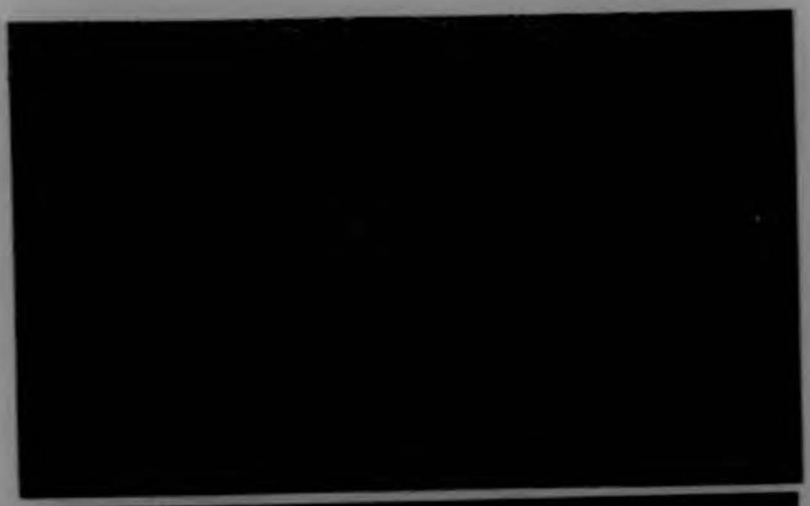

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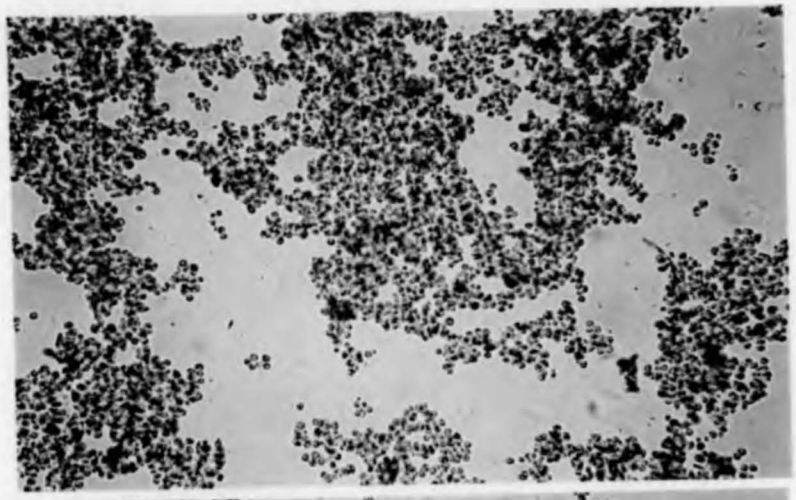

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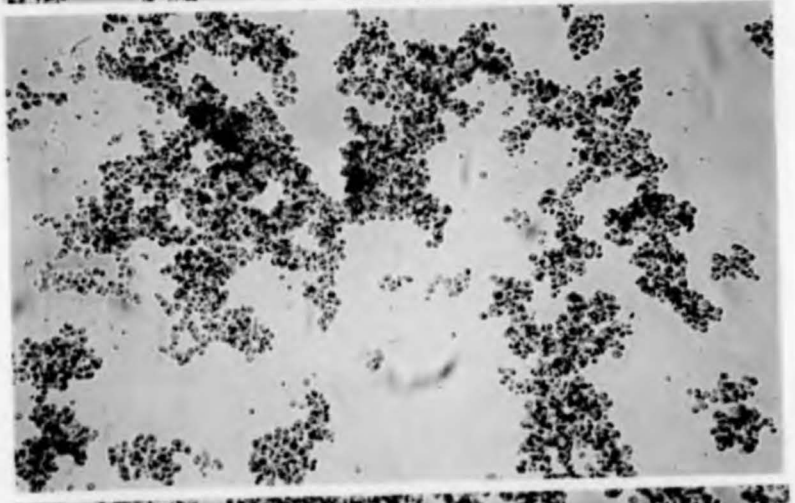

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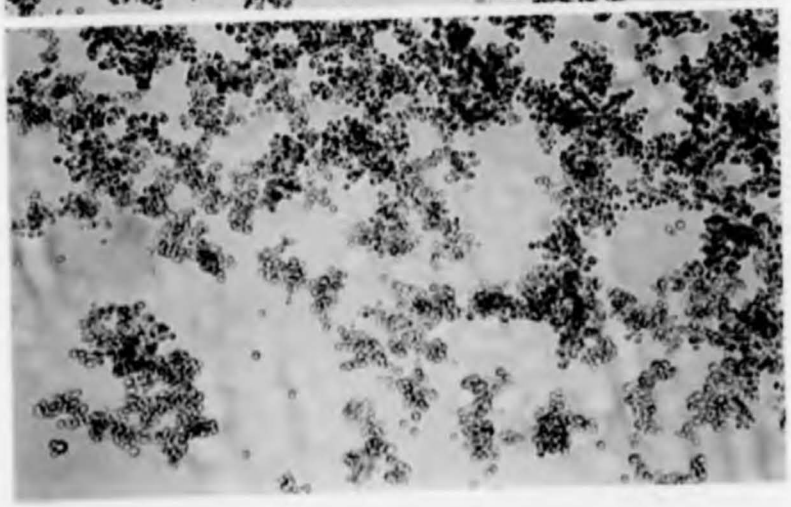

Figure 102: Photomicrograph (x 100) showing concanavalin A-induced agglutination (25 µg/ml) of T. vaginalis (No.1315 (Tv10)). Many isolated organisms interspersed with small clumps (10 - 20 organisms/ clump) are visible.

Figure 103: Photomicrograph showing small clumps (10 - 20 organisms/clump) of T. vaginalis (No 1315(Tv10)) induced by Concanavalin A (25 µg/ml). (magnification x 400).

Figure 104: Photomicrograph (x 100) showing control plate for Concanavalin A-induced agglutination. T. vaginalis (No. 1315 (Tv10)) in PBS, no agglutination.

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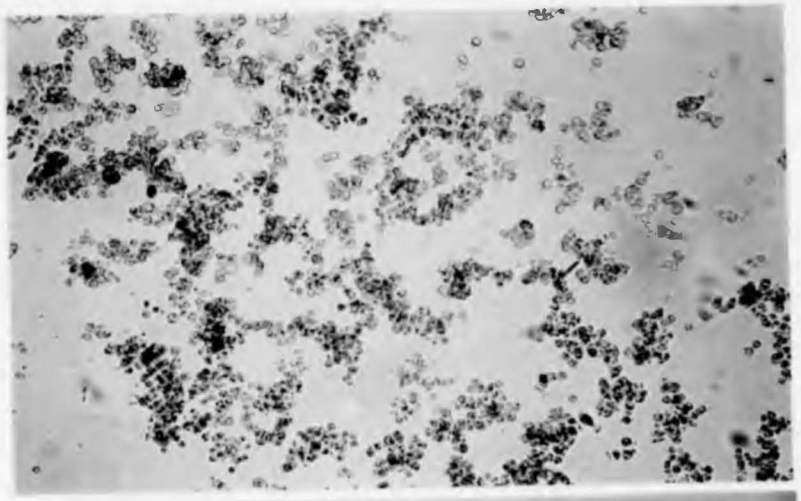

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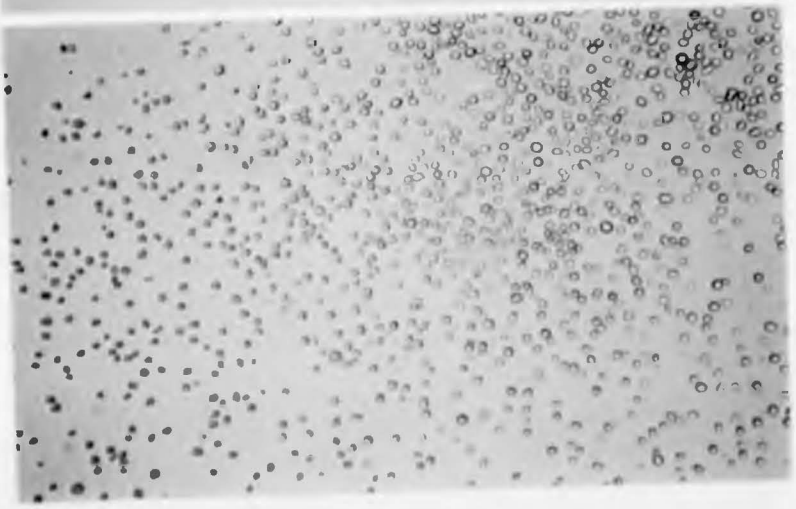

ABBREVIATIONS

ALD	aldolase
ATP	adenosine -5- triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid or acetate
G6PD	glucose -6- phosphate dehydrogenase
GPI	glucose phosphate isomerase
HK	hexokinase
IU	international unit
IUPAC-IUB	International Union of Pure and applied Chemistry and the International Union of Biochemists
LDH	lactate dehydrogenase
MDH	malate dehydrogenase
ME	malate dehydrogenase (decarboxylating) ($NADP^+$)
NAD	nicotinamide-adenine dinucleotide, oxidized form
NADH	nicotinamide-adenine dinucleotide, reduced form
NADP	nicotinamide-adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced form
PGM	phosphoglucomutase
Tris	2- amine -2- (hydroxymethyl) -propane - 1,3 diol

APPENDIX

Giemsa stain:

Stock Giemsa (filtered)	1 ml
Absolute alcohol	1.25 ml
Na_2Co_3 (0.5%)	0.1 ml
Distilled water	40 ml

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PART V

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