

ABSTRACT

Joanna Simons Tysor. Trichomonas vaginalis- An Amoeboid-Adherent Variant.
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Trichomonas vaginalis, first described by Donne in 1836, is a human, urogenital, sexually transmitted, protozoan parasite. Although usually ovoid-to-ellipsoidal and active, amoeboid-adherent forms have been described that are nonmotile, have short cytoplasmic pseudopodia, have missing or sluggish flagella, and have a tendency to stick to adjacent surfaces. Through a cloning technique, amoeboid-adherent strains that remain stable through subculturing have been isolated. In this study, the ovoid-motile strain is compared to the amoeboid-adherent strain via light and electron microscopy, analysis of surface proteins, comparison of colonial morphology, analysis of growth rates, and lactic acid production.

Except for cell shape the ovoid-motile and amoeboid-adherent forms were found to exhibit similarities in organelle structure and arrangement. Their colonial morphology differs in that the amoeboid colonies adhere to each other to form pinpoint colonies while the motile forms demonstrate diffuse growth throughout the culture tube. The banding from protein analysis varied slightly suggesting a possible difference in the surface proteins.

TRICHOMONAS VAGINALIS-
AN AMOEBOID-ADHERENT VARIANT

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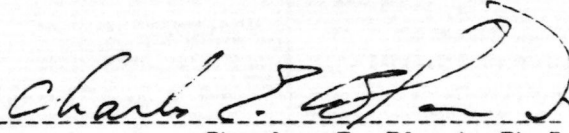
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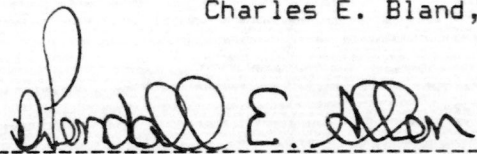
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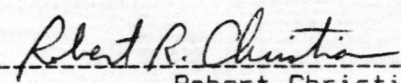
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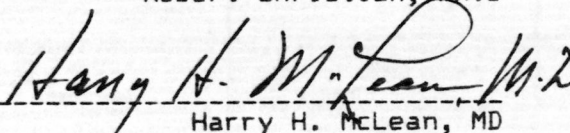
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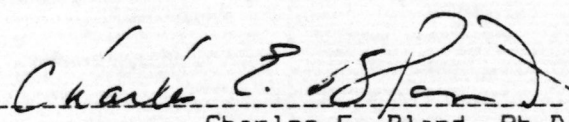
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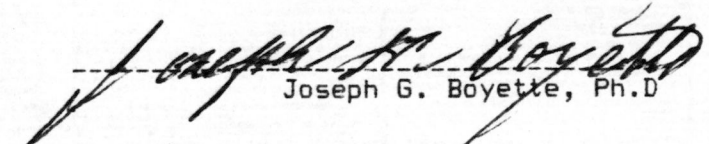

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DEDICATION

I would like to dedicate this work to my husband, Michael, who has given me support and encouragement in all my endeavors.

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ABBREVIATIONS

a	axostyle	
af	anterior flagella	
c	costa	
cv	collapsed vesicle	+CHO carbohydrate rich medium
dm	electron dense material	-CHO carbohydrate deficient medium
er	endoplasmic reticulum	
g	Golgi body	
gg	glycogen granule	
h	hydrogenosome	
k	kinetosome	
lm	limiting membrane	
ml	marginal lamella	
n	nucleus	
p	pelta	
ps	pseudopodium	
r	ribosome	
rf	recurrent flagella	
s	striated rootlet	
um	undulating membrane	
v	vacuoles	

INTRODUCTION

Trichomonas vaginalis (Phylum, Protozoa; Class, Mastigophora; Order, Trichomonadida; Family, Trichomonadidae) was described initially by Alfred Donne in 1836 from a human vaginal exudate. Since that time, many researchers have conducted morphological, cultural/physiological and pathological studies on this urogenital pathogen of humans (Bland, 1932; Powell, 1936; Wenrich, 1943,1947; Howes, 1947; Jirovec and Petru, 1968; and Honigberg, 1978). A review of these findings follows:

MORPHOLOGY

Early investigators using light microscopy described T. vaginalis to be typically ovoid-to-ellipsoidal, and to have four anterior flagella, an undulating membrane, and a posterior appendage, the axostyle. Internal structures described included an anterior nucleus, chromatic granules, and some vacuoles (Honigberg and King, 1964). These studies used stained specimens and living organisms observed with phase contrast microscopy.

The first electron microscope studies (Inoki et al., 1960; Ying et al., 1966; Smith and Stewart, 1966) added ultrastructural information to light microscope studies. Nielsen et al. (1966) with improved fixation and embedding techniques, were more accurate in their early accounts of T. vaginalis fine structure. Major features disclosed in this study include: a triple-layered limiting membrane; an axostyle composed of parallel tubular fibers extending from the anterior to the tip of the caudal axostylar extension; an anterior parabasal body located opposite the axostyle; five kinetosomes and their associated typical eukaryotic flagella; two rootlet fibers, the costa and parabasal filament, both cross-striated and attached

to the kinetosomes; a finlike undulating membrane with an attached flagellum; variably located rough endoplasmic reticulum; vesicles arising from membrane invagination or from the parabasal body; and chromatic granules in close association with the axostyle or costa, named respectively the para-axostylar and para-costal granules. Other fine structural studies investigated the nature of the chromatic granules (Filadora, 1970; Muller, 1980), also referred to as the hydrogenosome (Benchimol and De Souza, 1983), compared in vivo and in vitro cells (Nielsen, 1975), examined the interaction of T. vaginalis with vaginal epithelium (Nielsen and Nielsen, 1975), and used a freeze fracture technique (Honigberg et al., 1984) which elucidated membrane composition in T. vaginalis.

CULTURE MEDIA DEVELOPMENT

Early efforts to cultivate T. vaginalis, in vitro, began in the 1900's (Boeck and Drbohlav, 1925; Johnson, 1942; Lynch, 1915; Bland, et al., 1932). Trussell (1940) was the first to isolate a bacteria-free culture of T. vaginalis using 1% liver infusion agar and 5% placental extract. However, it was not until the use of antibiotics that pure cultures could easily be obtained (Adler and Pulvertaft, 1944; Johnson et al., 1945). Johnson and Trussell (1943) devised a complex medium of human serum and cystein-peptone-liver infusion-maltose (CPLM) to which penicillin (500-1000 units/ml) was added. In further studies on nutritional requirements by Johnson (1947) the liver infusion was replaced with a mixture of growth factors (ascorbic acid, glutamic acid, choline, folic acid, and xanthopterin). This resulted in a stimulation of cell multiplication. A Complete Trypticase Nutrient Medium developed by Sprince and Kupferberg (1948) for T. vaginalis replaced peptone

with trypticase. Cell multiplication proved to be less abundant than for CPLM medium of Johnson and Trussell (1943), but the trichomonads were active and more uniform in size and appearance (Sprince and Kupferberg, 1948). Kupferberg et al. (1948) simplified this complex medium in an effort to recognize the essential growth factors and make the medium less expensive and easier to make. The elimination of nonessential components resulted in a Simplified Trypticase Serum (STS) medium.

During the 1950's, the media used for T. vaginalis cultures was monophasic, liquid or semisolid, or diphasic, having an agar slant with a fluid overlay. The first to cultivate T. vaginalis on the surface (plate culture) or within (stab culture) solid medium (.5-2% agar) were Asami et al. (1955). This important achievement was succeeded by pour plate methods (Samuels, 1962; Hollander, 1976), some of which were utilized for antibiotic assays (Filadoro and Orsi, 1958; Samuels and Stouder, 1962). Ivey (1961) utilized a pour plate technique (.35-.55% agar) to isolate clones of T. vaginalis which could be serially transferred and maintained in order to study strain variation.

The CPLM medium and the STS medium, described earlier, were modified by Diamond (1957) to produce a Trypticase-Yeast Extract-Maltose (TYM) medium which was employed in some pour plate methods (Ivey, 1961; Samuels, 1962; Hollander, 1976). The development of culture media for T. vaginalis was furthered by Hollander and Frost (1965) when ascorbic acid was found to be the reducing agent of choice over cysteine and thioglycollate. Ascorbic acid was previously known to be a growth stimulant, but a reason was not known (Johnson, 1947). Bromke (1986) developed a serum-free, lipid supplemented medium for T. vaginalis which compared favorably to Diamond's

TYM medium.

A modification of Diamond's TYM medium (Hollander and Leggett, 1985) was found to yield excellent growth. The modifications were that cysteine was replaced with ascorbic acid as the reducing agent, Sorenson's buffers with potassium salts were included, and Vitamin B₁₂ was added.

PHYSIOLOGY

T. vaginalis has been described as facultative or aerotolerant anaerobe (Lindmark and Muller, 1973; Mack and Muller, 1980) which utilizes glucose or polysaccharides composed of multiple glucose units (Kupferberg et al., 1953). The endogenous glycogen reserves and exogenous carbohydrate sources oxidize incompletely to produce organic end products, CO₂, and H₂ (Steinbuchel and Muller, 1986b). Lactic acid has been identified to be the major acidic end product of metabolism (Wellerson and Kupferberg, 1962; Jirovec and Petru, 1968). The enzymes catalyzing the breakdown of glucose to pyruvate and lactate are located in the cytosol and the conversion of pyruvate into acetate, H₂, and CO₂ occurs in the hydrogenosomes (chromatic granules) (Muller, 1980; Steinbuchel and Muller, 1986a).

AMOEBOID MORPHOLOGY

It has been known for some time that T. vaginalis has the ability to vary its size and shape; a feature attributed to environmental conditions and the growth phase. Honigberg and King (1964) observed pseudopodia formation in freshly isolated strains. The fine, posterior, pseudopodia-like extensions were used for attachment while feeding, and they were present in most strains. Freshly isolated cultures of some strains contain

organisms which appear amoeboid, a condition considered to be a degenerative change (Honigberg and King, 1964). These amoeboid-adherent (AA) forms are nonmotile with inactive or absent flagella and adhere to glass or other surfaces via their fragile irregular limiting membrane. The amoeboid cells have been observed to be replaced by round-to-ovoid organisms when the amoeboid forms are maintained in Diamond's TYM medium (Nielsen, 1975). This was thought to be due to a nutritive deficiency.

PATHOLOGY

In tissue culture, amoeboid strains of T. vaginalis were reported by Nielsen and Nielsen (1975) to adhere to the tissue and subsequently invade the host cells. The mechanism of host cell injury by T. vaginalis has been described as being from direct contact, by damage from toxic secretions, or both (Honigberg, 1979).

Pathogenicity studies of T. vaginalis have utilized mice as experimental hosts following intraperitoneal, intramuscular, and subcutaneous inoculations (Honigberg, 1979). The lesions produced were histologically studied for pathological changes in relation to the severity of the clinical infection. A standardized pathogenicity test, "The Mouse Assay", in which subcutaneous lesions were measured to determine strain virulence was developed by Honigberg, et al., 1966. A correlation between the size of the abscess caused by the parasite in the experimental mouse host and the degree of pathogenicity seen in the infected natural human host was observed by Frost and Honigberg (1962). In other studies, cell cultures were used to observe the pathogenic effects of T. vaginalis via light microscopy and scanning electron microscopy (Kulda, 1967; Heath, 1981). In

a study by Farris and Honigberg, 1970, the more pathogenic strains were amoeboid, tending to adhere to cell cultures, while mild T. vaginalis strains moved independently and with less interaction (Farris and Honigberg, 1970). Frequent passage in nonliving medium has been shown to cause a loss or decrease in virulence (Honigberg, 1978). A relationship between pathogenicity and the in vitro growth rate of cultured T. vaginalis strains was determined to be inversely proportional (Honigberg, 1961), and evidence correlates the amoeboid trait with pathogenicity (Rasmussen et al., 1986).

PURPOSE

It is the purpose of this study to compare and contrast two strains of T. vaginalis, a normal ovoid-motile (OM) strain and an adherent-amoeboid (AA) strain. Both have been successfully isolated and maintained in a modified Diamond's TYM medium for over 150 passages. The amoeboid isolate in pure culture has not been available for study until recent isolation of the AA variant of T. vaginalis by Hollander and Tysor (in press). A comparison study was initiated with an interest in isolating specific differences between the two strains of T. vaginalis because of their obvious morphologic differences. Studies to compare the AA and OM strains involved the following: colonial morphology in culture tubes and pour plates; growth rates; light microscopy; transmission and scanning electron microscopy; protein variation in polyacrylamide gel electrophoresis; and gas chromatography to measure lactic acid as a toxic end product.

MATERIALS AND METHODS

ORGANISMS AND GROWTH CONDITIONS

Trichomonas vaginalis strains B₁-B₁₇, B₂₀, and B₂₂-B₂₆ were obtained from Dr. Harry McLean (East Carolina University Student Health Clinic, Greenville, North Carolina), strains B₁₈ and B₁₉ were contributed by Dr. Carl Mattern (Baltimore, MD), and strain B₂₁, Honigberg's amoeboid strain JH30A, was purchased from the American Type Culture Collection (ATCC # 30235). These strains were maintained in modified Diamond's Trypticase-Yeast extract-Maltose (TYM) medium modified with ascorbic acid and also in a carbohydrate deficient modification of this medium. The carbohydrate medium (+CHO) contained: Trypticase (20 g), Yeast extract (10 g), Ascorbic acid (1.0 g), KCl (1.0 g), KHCO₃ (1.0 g), K₂HPO₄ (0.5 g), KH₂PO₄ (1.0 g), FeSO₄ (0.1 g), Vit. B₁₂ (.004 g (4 mg)), Maltose (5.0 g), Ion agar (0.5 g (.059%)), and tapwater (850 ml). The ingredients were heated to boiling, dispensed in 6.2-ml quantities into 13mm X 100mm, screw-capped test tubes, autoclaved for 15 min at 15 psi and 121°C, then cooled and stored under refrigeration (4°C). Just prior to inoculation, heat-inactivated calf serum (.9 ml), crystalline penicillin G potassium (5000 units/ml) and streptomycin sulfate (1000 ug/ml) were added to each tube. The carbohydrate deficient medium (-CHO) was identical to the modified TYM medium except for the absence of maltose. The pH was approximately 6.8 and was not adjusted. Cultures grown in +CHO media were transferred 3-4 times per week, and cultures grown in -CHO media were transferred weekly. Cultures in both media

were incubated at 37°C.

Duplicate cultures of all strains were stored frozen with 10% dimethylsulfoxide (DMSO, 0.1 ml/tube), a freeze protectant, at -70°C in a Queue Cryostar freezer (Lumsden et al., 1966).

CLONING ISOLATES

This method was used to isolate individual clones from wild type culture strains. The ingredients of the medium were identical to the cultivation medium, with the exception of an increase in agar concentration to 3.6 g/850mls (.42%). The initial ingredients were autoclaved 15 minutes at 15 psi and 121°C and then cooled in a 40°C waterbath. The medium was dispensed in 25 ml quantities into sterile 50 ml flasks, with the addition of 3 ml heat-inactivated calf serum and antibiotics (crystalline penicillin G potassium (5000 units/ml) and streptomycin sulfate (1000 ug/ml)). A suspension of inoculum, serially diluted ten-fold with 1N KCL, was then added to the medium, mixed and poured into a sterile 100 x 15 mm petri plate. The plates were incubated right side up because of the low agar concentration. To absorb the excess moisture, filter paper (Whatman No. 4) was placed in the cover of the petri plates prior to their sterilization. The plates were incubated under anaerobic conditions in GasPak anaerobic jars (BBL, Cockyesville, MD) at 37°C for 7-10 days. Isolated spherical colonies were selected and observed microscopically for colonial morphology (Olympus Stereoscope SZ H equipped with a transilluminator base and camera) and also using darkfield wet mount preparations. The selected colonies were then inoculated into liquid media (+CHO and -CHO), labeled and incubated

(37°C) to further observe the morphology of the clones. Wild type strains B₇ and B₁₈ were cloned to produce pure strains B₁₈C₉, an ovoid-motile (OM) clone and B₇C₂, an amoeboid-adherent (AA) clone. This cloned strain (B₇C₂) was recloned by the pour plate technique to insure its purity. This produced a stable AA isolate, B₇RC₂B. These two cloned strains (B₇RC₂B and B₁₈C₉) were utilized most in the comparison study.

LIGHT MICROSCOPY

Daily observations of wet mounts of organisms were made using a Leitz HM-LUX microscope with a darkfield condenser. A Nikon Microphot-FX with Planapo objectives and Differential Interference Contrast (DIC) was utilized for photomicrographs. In addition to observations of living specimens, some preparations were fixed with 4% osmic acid vapors for 1-3 minutes.

To stain flagella, smears prepared from cultures were air dried and stained using a modified Rhodes Silver-Plating flagellar stain. After the smear air-dried, the slide was flooded for four minutes with a mordant solution (aluminum potassium sulfate (25 ml), 10% tannic acid (50 ml), and 5% ferric chloride (50 ml)). After four minutes, the smear was rinsed gently in distilled water, and flooded with the stain solution (5% silver nitrate (90 mls), and concentrated ammonium hydroxide (2-4 mls)). The flooded slide was placed on a warming tray (40°C) for four minutes. The stain was rinsed off the slide with distilled water, and the slide was allowed to air dry.

SCANNING ELECTRON MICROSCOPY

An 8-chamber Lab-Tek Chamber slide containing +CHO media (Miles Scientific, Naperville, Il) was inoculated with 24-48 hr cultures (B₂₂, B₂₁, B₂₀ and B₂₁, B₁C_{2A}, B₇RC_{2B}, B₁₈C₉, B₁₈C₄, and B₁₈C₈). The slide growth chamber was then incubated under anaerobic conditions using the BBL GasPak System at 37°C for 48 hrs. The media in the individual chambers was gently removed with a pipet and replaced with Phosphate Buffer (pH 7.4). This washing was repeated three times for 15 minutes. The cells were fixed with a modified Karnowsky's fixative (4% Paraformaldehyde, 2.5% Glutaraldehyde) pH 7.2, for 1 hr. After washing three times for 15 minutes each in Phosphate Buffer, the cells were post-fixed with 2% osmic acid in Sorensen's Phosphate Buffer for 1 hr, then washed in Phosphate Buffer (3 X 15 min). The chambers and rubber gasket were removed from the slide. The cells were then dehydrated in a graded ethanol series (30% ETOH (10 min), 50% ETOH (10 min), 70% ETOH (10 min), 95% ETOH (10 min X 2) and 100% ETOH (overnight)). To facilitate placing the slide in the chamber of the critical point dryer, the slide was etched with a diamond scribe and broken in half. After critical point drying in a Balzers CPD-20, the specimens were coated with approximately 90 nm of gold palladium (Anatech Hummer V Sputter Coater), and observed with an International Scientific Instrument-40 Scanning Electron Microscope (10 Kv).

TRANSMISSION ELECTRON MICROSCOPY

AA (B₇RC₂B) and OM (B₁₈Cg) strains were cultured in media (+CHO and -CHO) with and without agar for two passages. Cultures (24-48 hr) were fixed by the addition of 2.5% Glutaraldehyde and .075 M Phosphate Buffer (pH 7.2) in 2X volume to each test tube for 15 minutes. Cultures were then transferred to centrifuge tubes, centrifuged 15 min and washed with Phosphate Buffer (pH 7.2) three times. Whatman No. 4 filter paper was trimmed to fit lengthwise into a centrifuge tube. Each tube was placed on its side after the last centrifugation and the filter paper was inserted touching the pellet. The excess moisture from the pellet was absorbed for 15 min, but the pellet was not allowed to dry completely. Molten agar (2%), cooled to 40°C, was added to the pellet and mixed gently. After solidification, the pellet was dislodged and cut into small cubes, washed 2X in Phosphate buffer for 15 min, and stored overnight in Phosphate Buffer. The cubes were washed in Phosphate buffer for 10 min, postfixed in 1% OsO₄ for 1.5 hrs, washed 2X in buffer for 10 min and dehydrated in ETOH (50%-15 min, 70%-overnight, 70%-15 min, 95%-15 min, 100%-15 min, 100% 1 hr). Infiltration proceeded with propylene oxide (PO) washings, 3X for 10 min, PO:Epon 812 (3:1, 3-4 hrs; 1:1, overnight; and fresh resin, 1 hr.). The cubes were then embedded in fresh Epon 812 and placed in a 60°C oven for 48 hrs. Blocks were trimmed and sectioned on a Reichert OM U2 ultramicrotome. The sections were pretreated with .5N HCl (1 min) then stained in uranyl acetate (10 min) and lead citrate (10 min). Observations were made on a Hitachi HS-8 transmission electron microscope.

MEASUREMENTS OF GROWTH RATE

AA and OM cultures (24-48 hrs) were inoculated into +CHO media and incubated at 37°C. Cell counts were made at 3 hr intervals for 24-36 hrs with a Spencer Bright-line Hemacytometer (1/10 mm depth). The generation times (G) were calculated by the standard formula

$$G = \frac{t \log 2}{\log b - \log a}$$

where t = the time interval between a and b; a = the number of trichomonads per milliliter at time a; and b = the number of trichomonads at time b.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Culture samples of AA (1.4×10^7) and OM (1.2×10^7), in rapid growth phase, were centrifuged for 10 min and washed in .075 M Phosphate buffer (pH 7.2) 3 times. The sediment was mixed with Treatment Buffer (SDS-4%, Glycerol-20%, Bromophenol blue-2-mercaptoethanol-10%, and .125 M Tris-Cl-pH 6.8) in a 1:1 ratio and boiled in a sealed capsule for 1 minute. The gels were run with 7.5% Polyacrylamide gel for 20 hrs at 5MA and 15% Polyacrylamide gel for 20 hrs at 10 MA using 5, 10, 15, 20, and 25 ul protein samples. Bovine serum albumin was used as the standard and gels were stained with Coomassie blue (1%).

GAS CHROMATOGRAPHY

Culture samples (48 hrs and 7 days) of AA and OM (+CHO and -CHO) were analyzed by Gas-Liquid chromatography by Dr. Robert Fulghum's laboratory, Department of Microbiology and Immunology, East Carolina University School of Medicine (Greenville, NC). The metabolic end products were recorded as a series of peaks in a chromatogram tracing. The peak heights (cm) for lactic acid were converted to milliequivalents/100mls with the aid of a standard curve.

SILICONIZED SLIDES

To insure that T. vaginalis does not adhere simply because of dirt contamination, a series of slides were silicon coated. Sigmacote (Sigma Chemical Company, St. Louis Mo.) was used to coat microscope slides and coverslips according to the manufacturer's instructions. Siliconized slides were used during wet mount observations of organisms on the light microscope.

RESULTS

COLONIAL MORPHOLOGY

Utilization of the pour plate cloning technique (Fig. 1)* made it possible to purify wild type T. vaginalis cultures into AA and OM clones. The isolated colonies were inoculated into culture tubes and grown to demonstrate colonial morphology (Fig. 2). In culture tubes OM strains displayed a diffuse growth pattern throughout the culture tube because of their active motility. The nonmotile AA strains adhered to each other forming pinpoint colonies. Usually, the wildtype was observed to be a combination of diffuse growth with pinpoint colonies.

The wild type isolates of T. vaginalis have been subcultured successfully in the modified version of Diamond's TYM medium for over 220 passages. The cloned isolates have remained stable for over 170 passages, during these comparison studies.

Figure 3 shows two colonies from a pour plate illustrating colonial differences. The size and margin variation were due to the location in the medium. The smaller, entire colony (A) was located within the medium and the larger, fuzzy colony (B) was a surface colony. When these two types of colonies were isolated and inoculated into medium there was no correlation between their morphology and strain type, OM or AA.

* NOTE: Figures begin on page 19.

GROWTH RATE

The growth curves of AA and OM strains (Fig. 4) indicate a small difference in growth rates. The range of generation time for OM (3.5-5.1 hrs) was slightly faster than that of AA (4.0-6.4 hrs.).

LIGHT MICROSCOPY

Photomicrographs illustrate the ovoid shape of OM strains (Fig. 5, 7, 9, 51) and the irregular shapes of AA strains (Fig. 6, 8, 10, 52). Chromatic granules are a prominent feature in phase-contrast micrographs (Fig. 7, 8) of AA and OM strains, and silver stain was employed to highlight flagella (Fig. 9, 10). The AA forms adhere to the slide surface creating the illusion that AA cells are larger than OM cells. The OM forms have little surface contact and are more compact. When siliconized slides were employed, AA forms would still adhere to the surface, ruling out adhesion due to dirt contamination.

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy enables one to observe surface morphology of OM (Fig. 11-13) and AA (Fig. 14-16) strains. The major surface structures, four anterior flagella, undulating membrane and recurrent flagella, and axostyle, are present in both strains with the exception of the adhesive pseudopodia in AA (Fig. 14-16). The anterior flagella and undulating membrane emerge anteriorly as the axostyle protrudes posteriorly.

TRANSMISSION ELECTRON MICROSCOPY

Other than the obvious differences in shape between the AA and OM strains (Figs. 17-20), fine structural comparison of the two showed no differences in internal structure. Because of this, the following description of internal structure is applicable to both strains.

The mastigont flagellar system is located anteriorly (Fig. 21-22) and consists of five kinetosomes and their associated flagella. All flagella exhibit the typical 9+2 arrangement of microtubules (Figs. 23, 25, 51, 52), and each terminates proximally in a basal body made up of nine triplets of microtubules. Short, nonstriated props radiate outward from each triplet of all basal bodies (Figs. 22, 36, 51, 52). Four of the basal bodies give rise to four, free, anteriorly directed flagella. The fifth basal body, angled slightly, is the base for the recurrent flagellum which is associated with an anterior-posterior, thin fold in the limiting membrane of T. vaginalis, the undulating membrane (Figs. 18, 24-25, 51-52).

A slight depression in the undulating membrane cradles the flagellum with no other apparent attachment (Fig. 24). The distal portion of the fold contains an electron-dense, accessory marginal lamella (Figs. 18, 25-26, 51-52) which runs the length of the membrane.

The axostyle, composed of a sheet of microtubules, extends from the anterior mastigont system to the tail-like posterior of the organism (Fig. 17, 35, 51-52). Each microtubule of the axostyle is attached to a delicate membrane (Fig. 31-32, 34). The anterior region of the axostyle, referred to in the literature as the pelta, encircles the mastigont organelles (Fig 17, 21-22, 26-28, 51-52) anterior to the nucleus. From the pelta, the microtubules of the axostyle proper then run posteriorly beside the nucleus

(Figs. 17, 27, 31, 51-52). Once past the nucleus, the microtubules coil before extending into the tail-like region (Fig. 17-18, 29, 32-35, 51-52). The number of microtubules in the axostyle is greatest at the junction of the pelta and axostyle (50-55) but decreases as it runs posteriorly to only a few in the tail.

Two or three striated rootlets originate in the vicinity of the mastigont system (Fig. 36-37, 51-52). These organelles are long and slender with uniform cross striations. One originates at the kinetosome of the recurrent flagellum (Fig. 21) and runs parallel to the undulating membrane. Other striated rootlets are associated with one of the kinetosomes of the anterior flagella and run between the Golgi body and nucleus.

The Golgi apparatus, referred to in the literature as the parabasal body, is located adjacent to the nucleus (Fig. 25, 41, 51-52). It is made up of a stack of flattened cisternae and associated vesicles. The vesicles increase in size as they near the cell periphery. Electron dense material is often found between the cisternae (Fig. 41).

Numerous membrane bound, electron-dense, granules (Fig 43) having a homogenous matrix are distributed throughout the organism, but are usually positioned around the axostyle (Fig. 18, 33, 35, 44) or a striated rootlet (Fig. 36, 43). From early light microscope studies these granules have been referred to collectively as chromatic granules or hydrogenosomes.

The cytoplasm of the T. vaginalis cell varies in its make up. Glycogen granules, found singularly or in rosettes (Fig. 44, 46-47) are distributed throughout the cell. Sparse rough endoplasmic reticulum surrounds the nucleus (Fig. 17, 20, 44) many free ribosomes occur throughout the cytoplasm. In some cases the ribosomes form a halo around the

hydrogenosomes (Fig. 45). Smooth endoplasmic reticulum (Fig. 17, 20, 47), food vacuoles (Fig. 17-20, 36, 40, 44, 46-47, 51-52) and what appear to be collapsed vesicles (Fig. 19, 42, 44, 46) are also distributed in the cytoplasm.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

From the SDS-PAGE analyzed whole cell suspensions of strains AA and OM, it was found that of the ten bands counted in the 7.5% polyacrylamide gel of the OM strain, 9 bands were seen in the AA strain (Fig. 48). Similarly, of the 23 bands of the OM strain in the 15% polyacrylamide gel, 22 bands were present in the AA strain (Fig. 49). Although banding patterns varied between AA and OM slightly they were similar enough to support the theory that these two strains are of the same species.

LACTIC ACID CONCENTRATION

The lactic acid concentrations of AA and OM cultures (48 hrs. and 7 days) are shown in Figure 50. The medium deficient in carbohydrates (-CHO) resulted in less lactic acid production than the +CHO medium.. The OM strain produced more lactic acid in the +CHO medium but the AA strain produced more in the -CHO medium over a 7-day period.

DIAGRAMMATIC REPRESENTATION

Diagrammatic representations (Fig. 51-52) of the OM and AA strains were reconstructed from light and electron microscope observations.

Fig. 1. Pour plate demonstrating isolated colonies used to produce pure clone cultures.

Fig. 2. Colonial morphology of culture tubes: B₇ (wild type parent culture); B₇RC₂B (AA clone); B₁₈C₉ (OM clone). Note pinpoint AA colonies, diffuse OM growth, and combined diffuse growth with pinpoint colonies in parent culture.

Fig. 3. Two colonies enlarged from pour plate demonstrating the difference in morphology. Colony A is located within the medium and colony B is a surface colony.

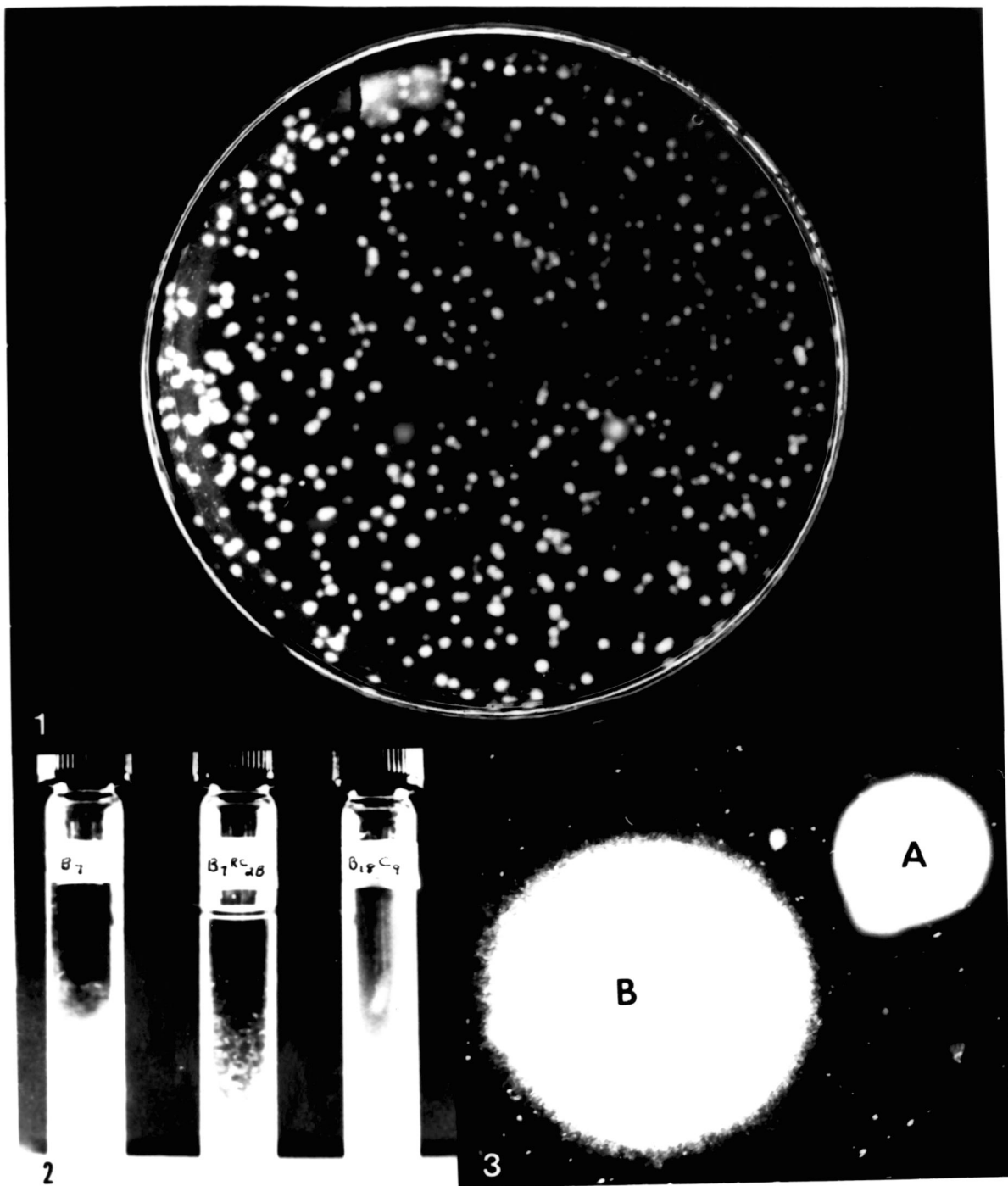


Fig. 4. Growth curves of AA (B₇RC₂B) and OM (B₁₈C₉) strains of T. vaginalis in carbohydrate rich medium (+CHO).

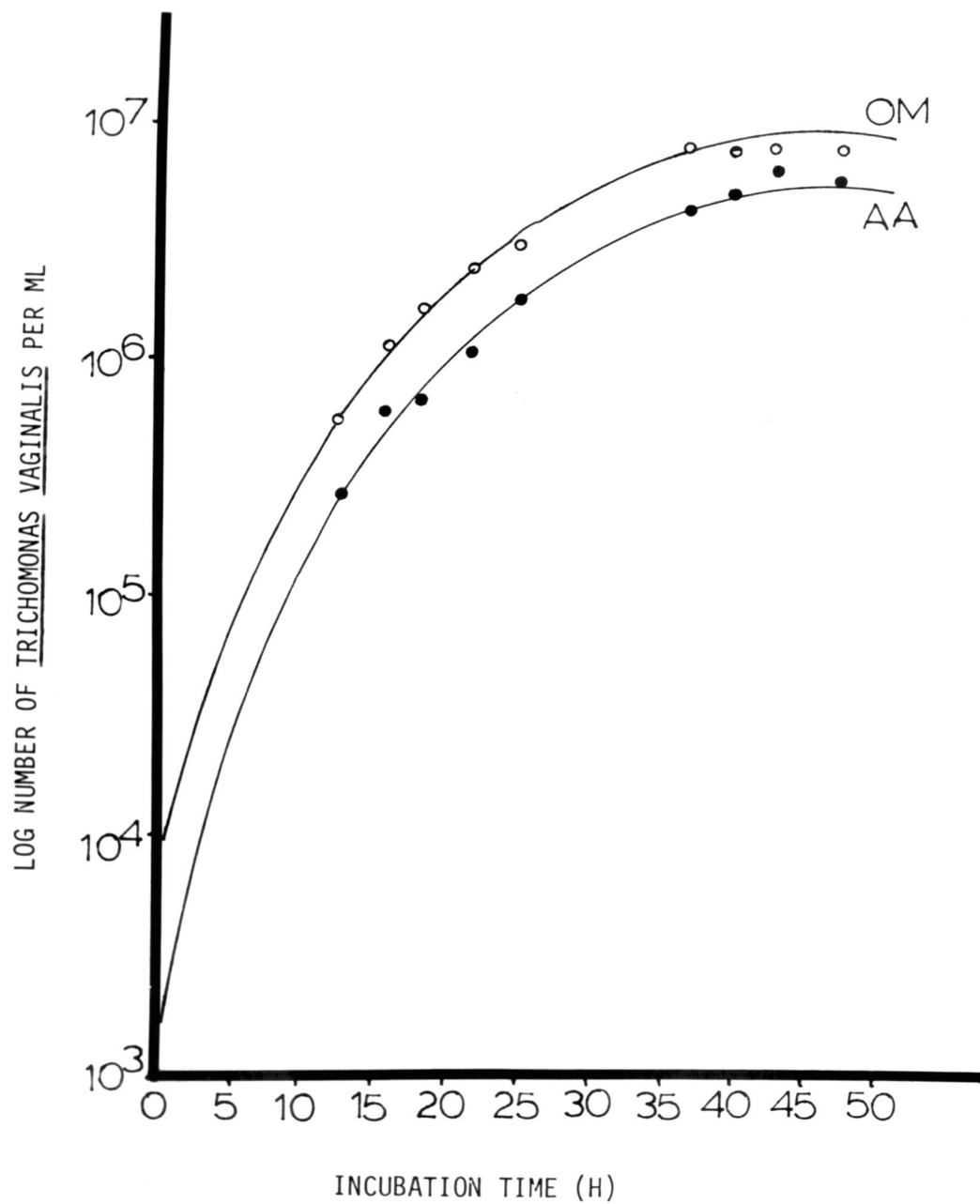


Fig. 5-6. Light micrographs using Differential Interference Contrast (DIC) of OM (Fig. 5) and AA (Fig. 6) strains. (625x).

Fig. 7-8. Light micrographs using Phase Contrast of OM (Fig. 7) and AA (Fig. 8) strains. Note the chromatic granules (arrow). (985x).

Fig. 9-10. Light micrographs using a modified Rhodes Silver-Plating Flagellar stain of OM (Fig. 9) and AA (Fig. 10). (1560x).

(bar = 10 μ m)

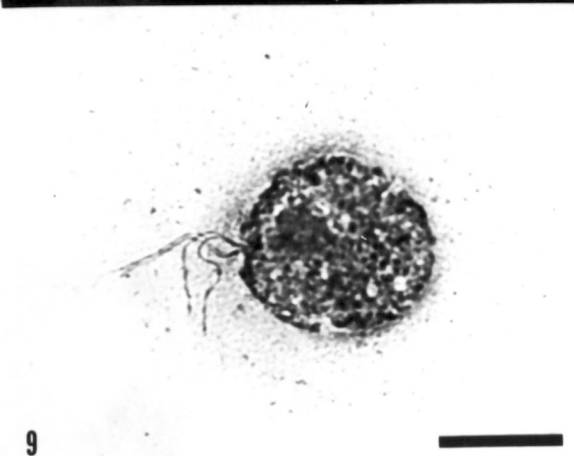
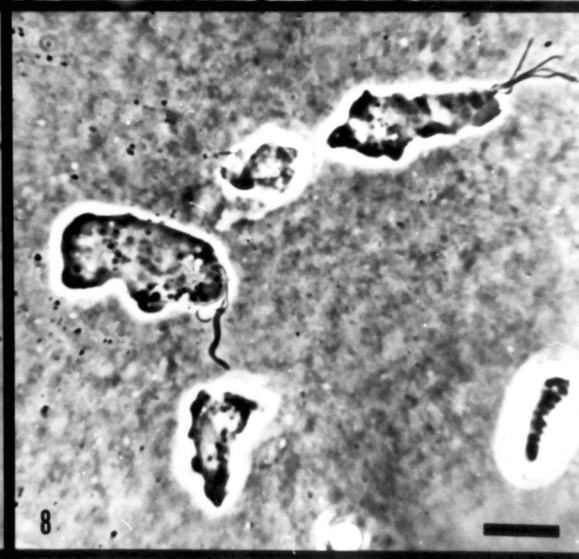
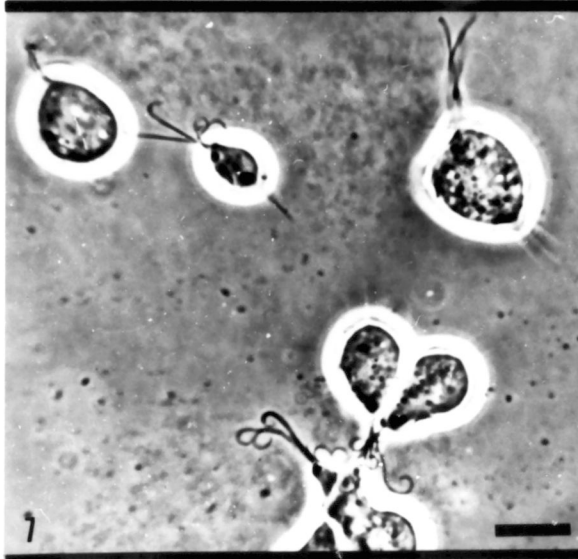
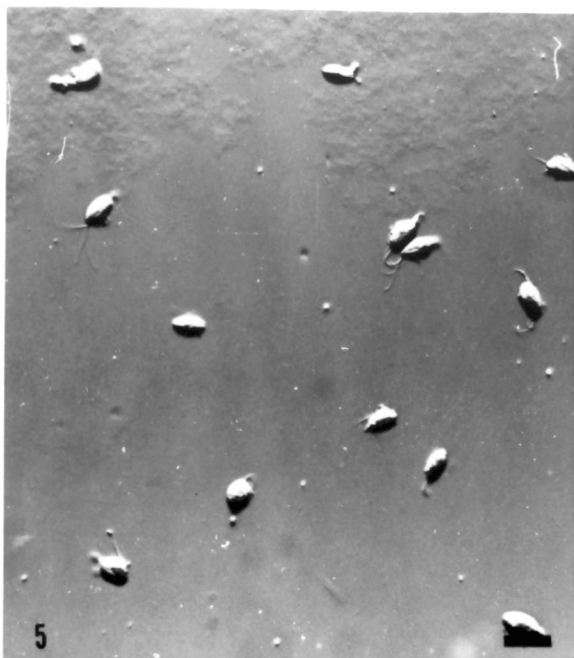
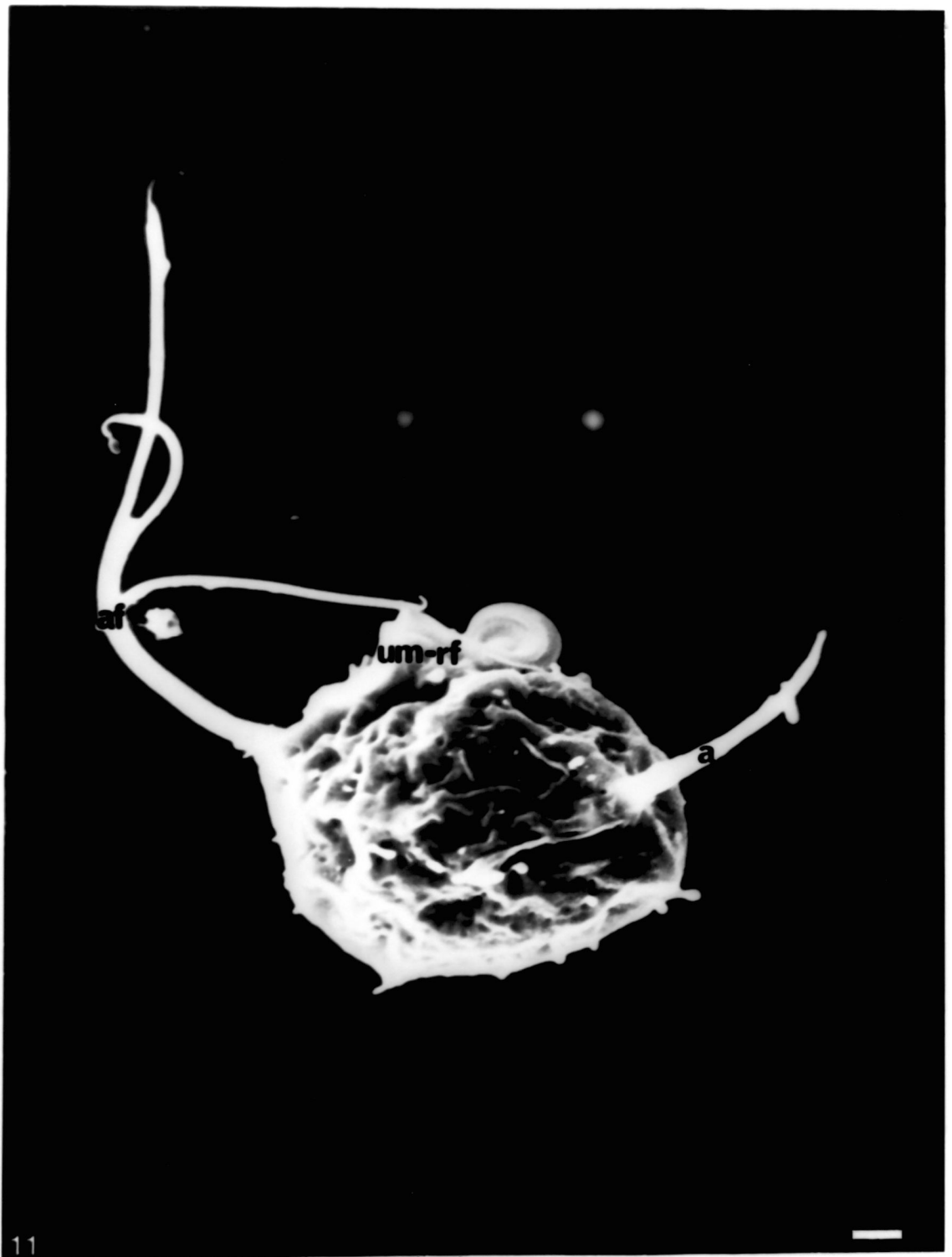


Fig. 11. Scanning electron micrograph (SEM) of OM T. vaginalis free swimming and ovoid. Axostyle (a), undulating membrane-recurrent flagellum (um-rf), anterior flagella (af). (8400x).

(bar = 1 um)



Figs. 12-13. Scanning electron micrographs of OM T. vaginalis, free swimming and ovoid.

Fig. 12. (3604x).

Fig. 13. (4209x).

(bar = 1 um)

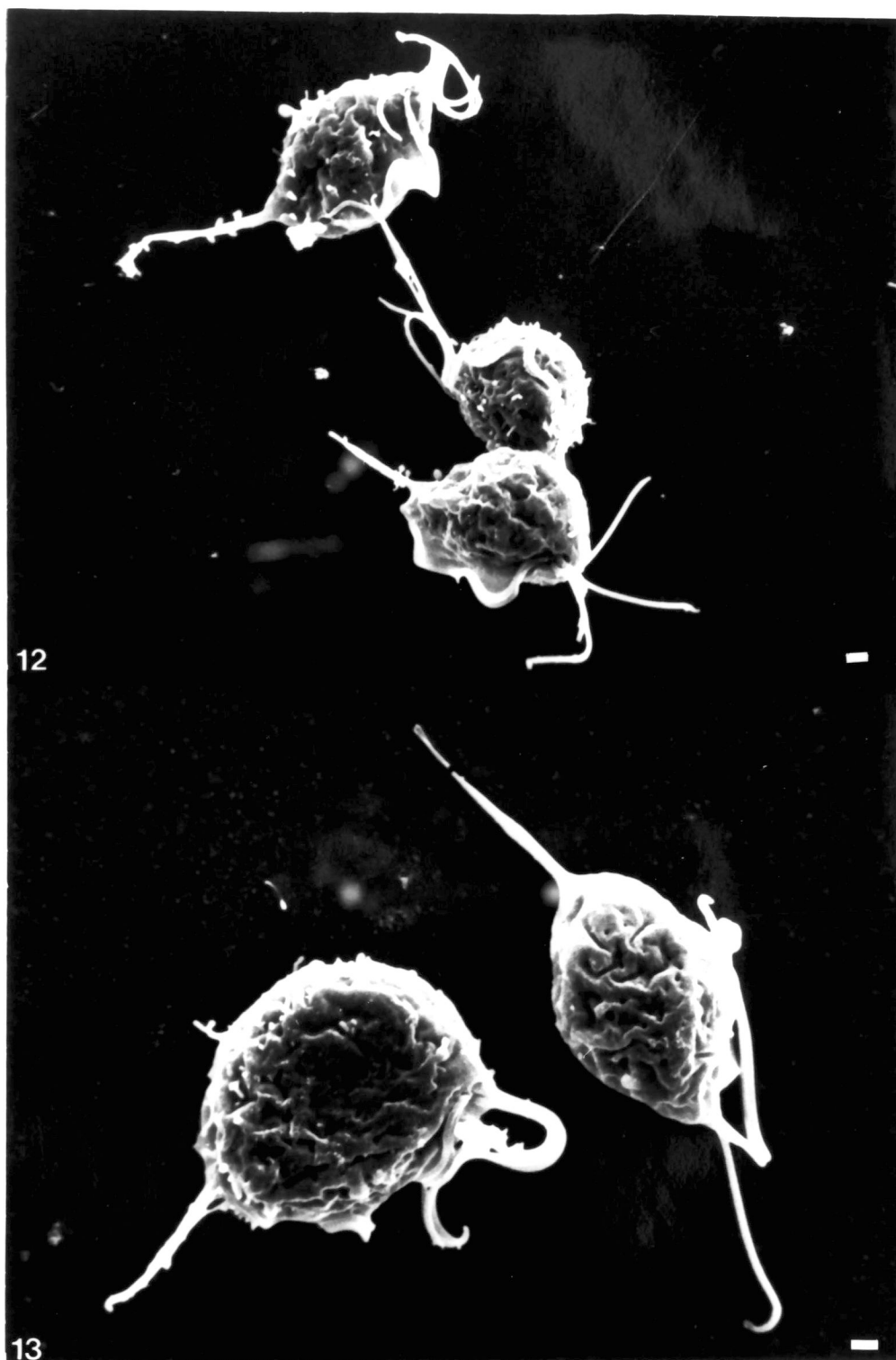


Fig. 14. Scanning electron micrographs of AA T. vaginalis adhering to the glass slide of the growth chamber. Note the irregular pseudopodia (ps) extensions. Axostyle (a), undulating membrane-recurrent flagellum (um-rf), anterior flagella (af). (8400x).

(bar = 1 μ m)

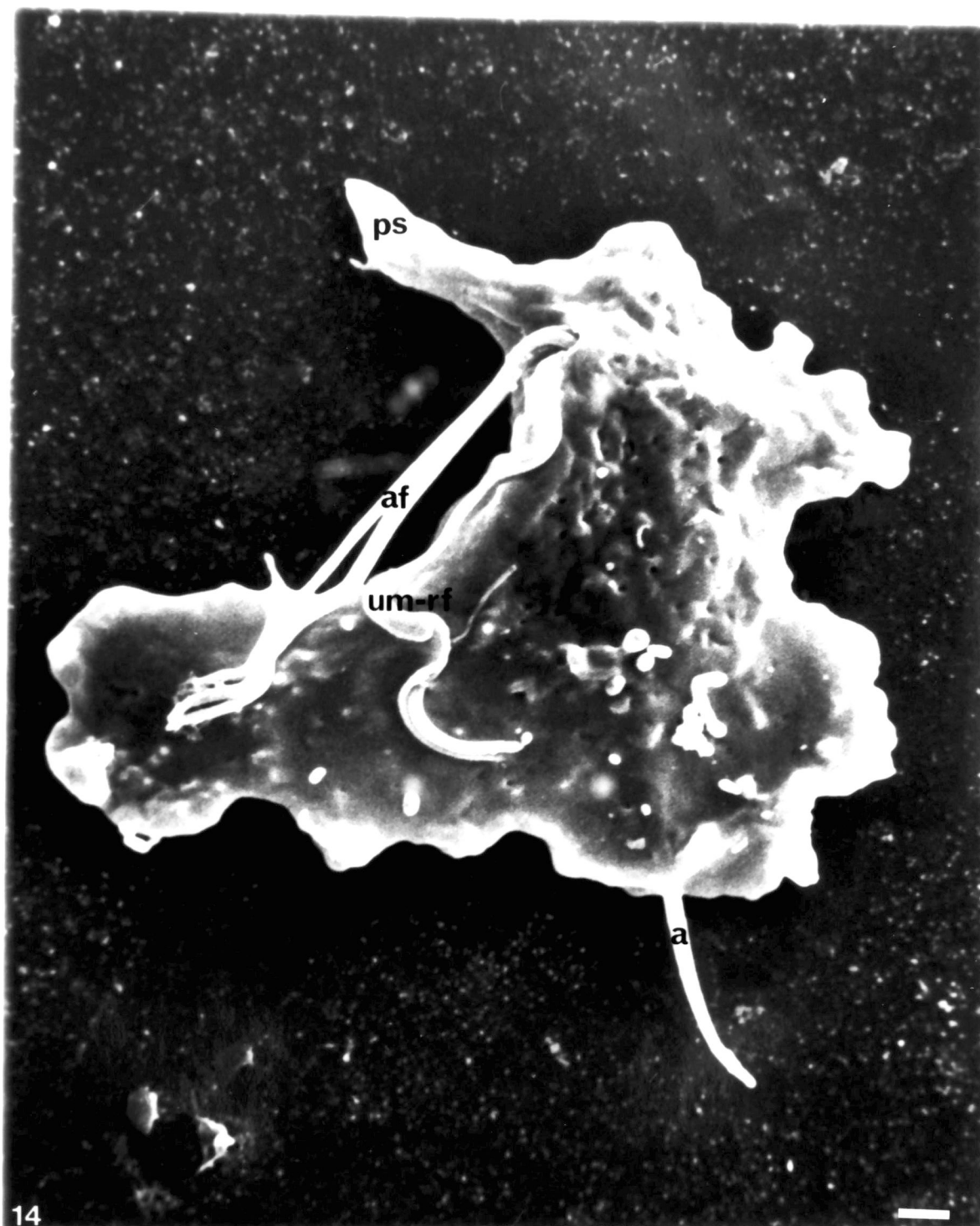
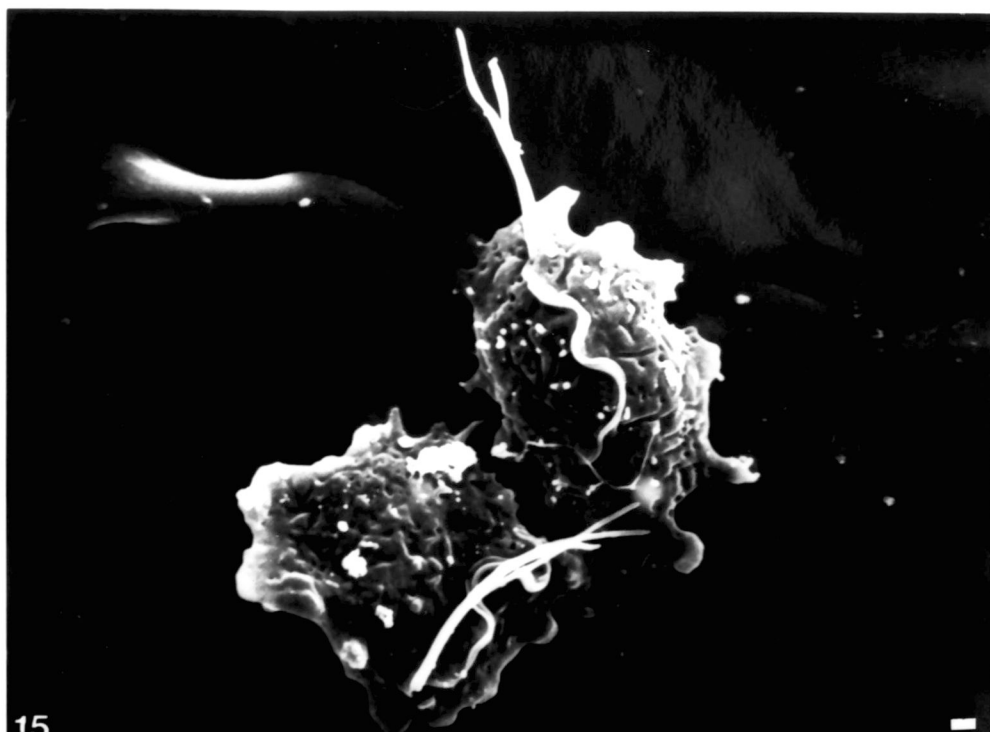


Fig. 15-16. Scanning electron micrographs of AA T. vaginalis adhering to the glass of the growth chamber.

Fig. 15. (3884x).

Fig. 16. (4655x).

(bar = 1 um)



15



16

Fig. 17. A transmission electron micrograph (TEM) of an OM T. vaginalis sectioned longitudinally. The anterior kinetosomes (k) are in cross-section, with a striated rootlet (s) extending from the kinetosome area. The pelta (p) encloses the kinetosomes and continues past the nucleus (n) as the axostyle (a). Chromatic granules, hydrogenosomes (h) surround the axostyle (a) and striated rootlet (s). The nucleus (n) is located anteriorly and food vacuoles (v) are distributed in the cytoplasm. Note the undulating membrane (um) and recurrent flagellum (rf) in cross section. (19,750x).

(bar = 1 um)

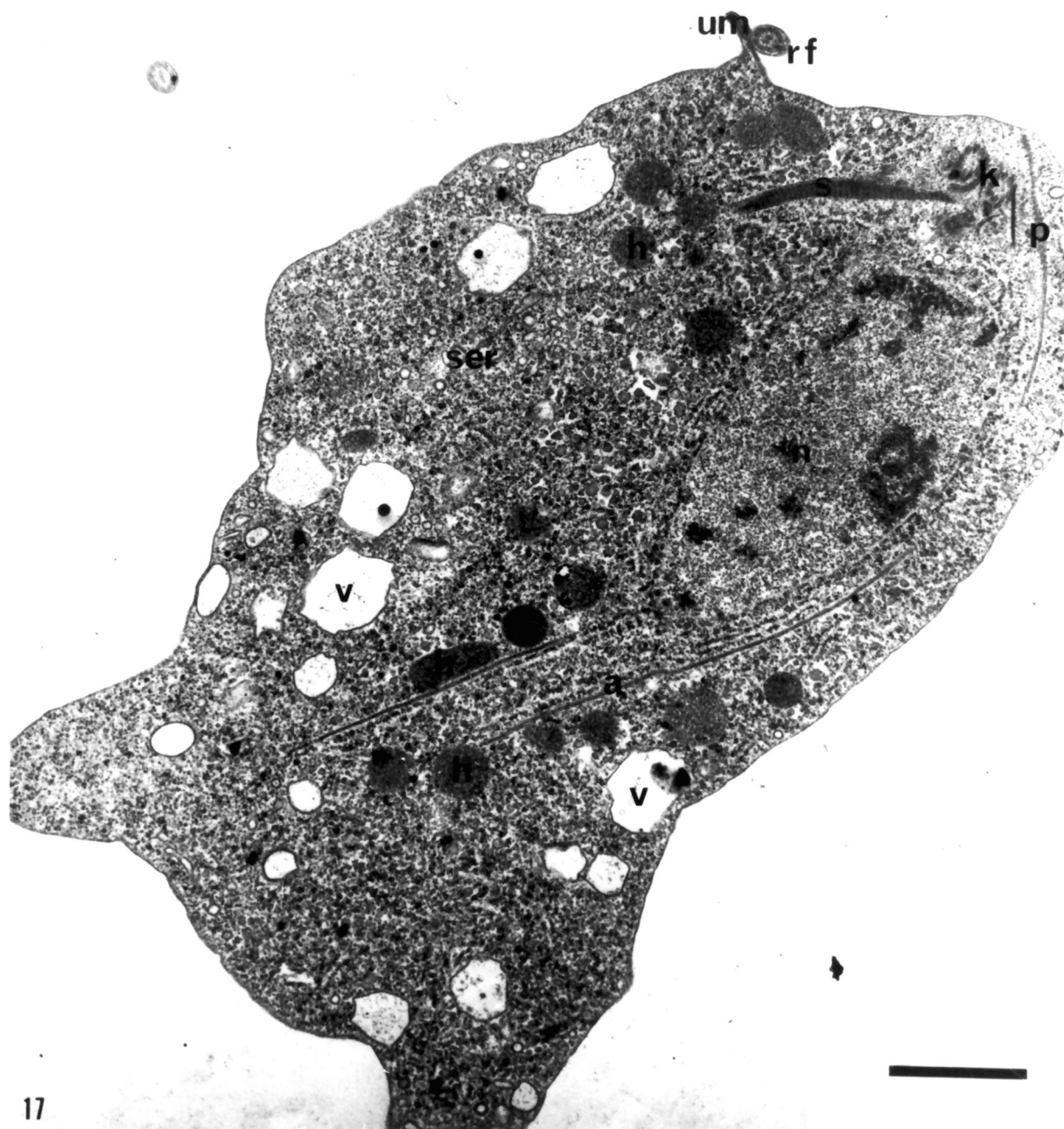


Fig. 18. A TEM of an AA T. vaginalis strain with pseudopodia (ps) sectioned longitudinally. The anterior flagella (af) are surrounded by the pelta (p). The pelta continues into the axostyle (a), with microtubules visible, and then extends posteriorly into an oblique section of the caudal axostyle (a). The undulating membrane (um), recurrent flagellum (rf), and one anterior flagellum (af) are cut at an oblique angle. The nucleus (n) is located anteriorly. The hydrogenosomes (h) surround the posterior portion of the axostyle and a striated rootlet (s). The cytoplasm contains smooth endoplasmic reticulum (ser), vacuoles (v), and small electron-dense glycogen granules (gg). Note the transition (arrow) from kinetosome (k) to flagellum (af) at the basal plate. (21,600x).

(bar = 1 um)

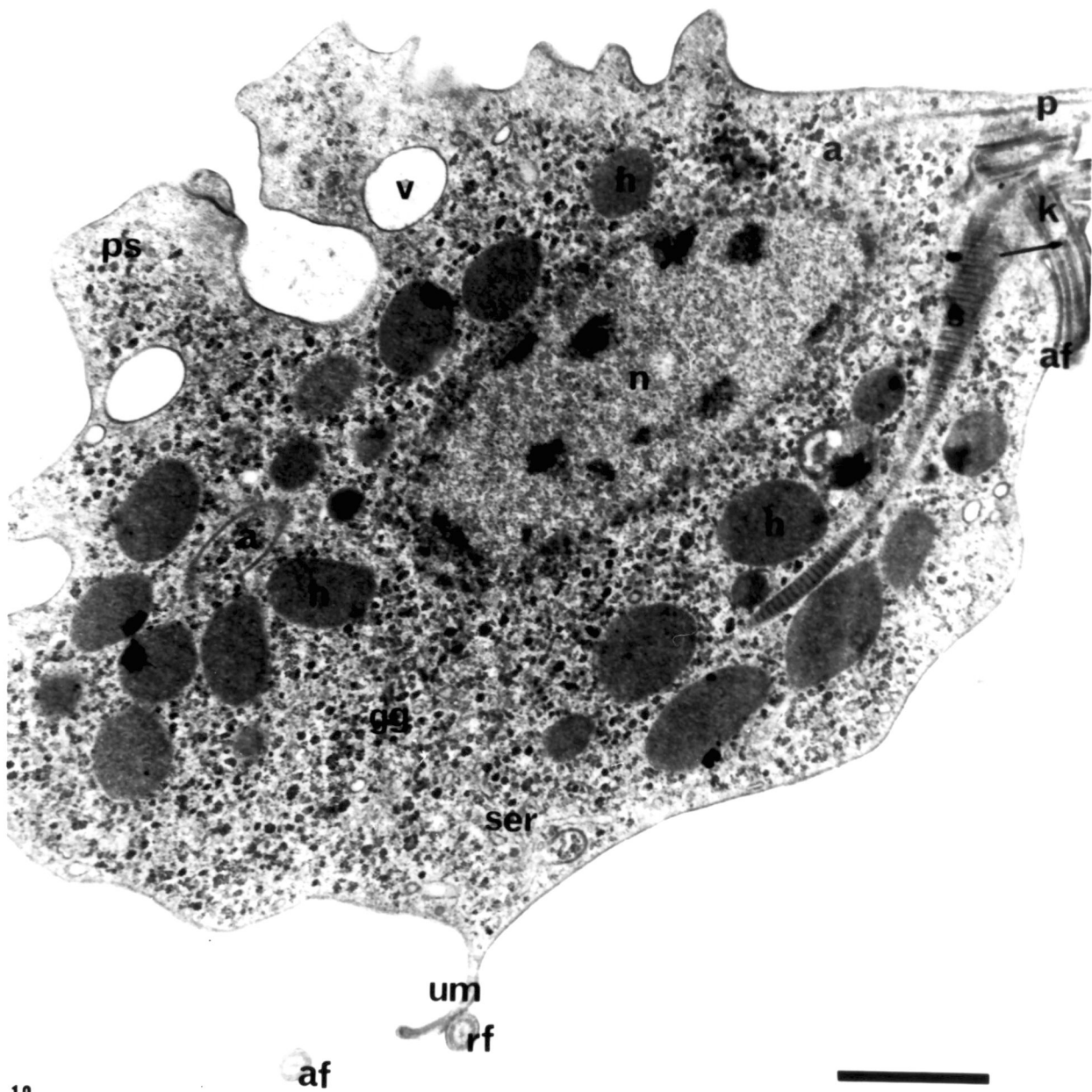


Fig. 19-20. Two examples of AA forms adhering to each other (Fig. 20) and a degenerative cell (Fig. 19).

Fig. 19. This posterior section contains an oblique cut of the axostyle (a), hydrogenosomes (h), vacuoles (v), a collapsed vesicle (cv), and glycogen granules (gg). (15,000x).

Fig. 20. The nucleus (n) is prominently surrounded by endoplasmic reticulum (er). The hydrogenosomes (h) are associated with a striated rootlet (s). (15,000x).

(bar = 1 μ m)

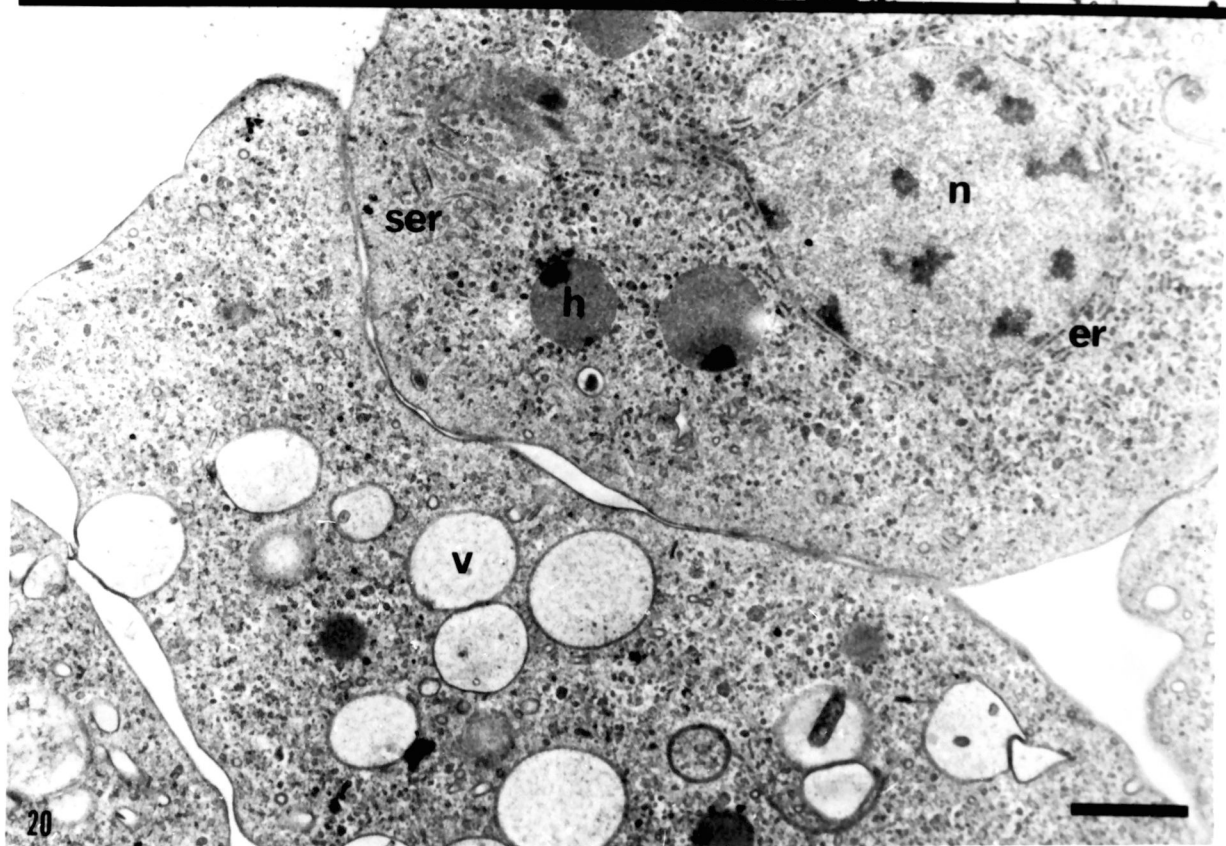
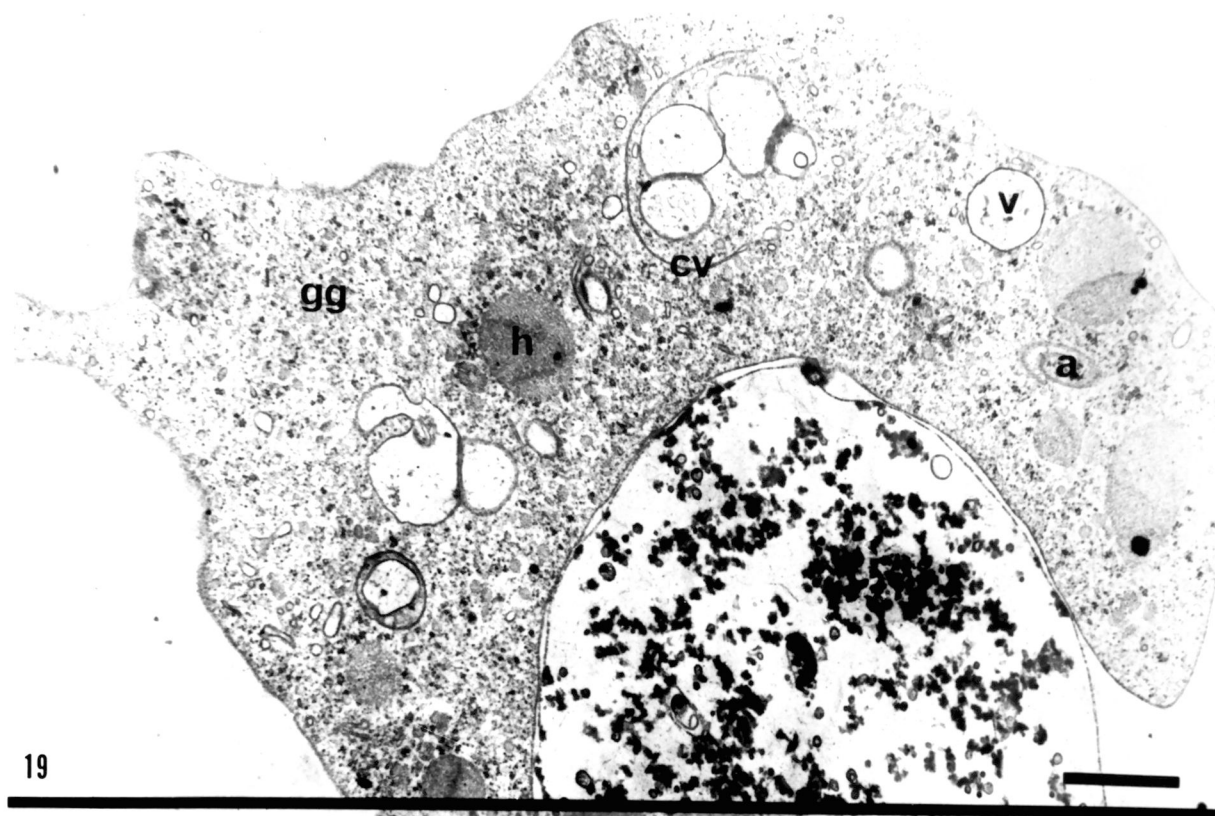


Fig. 21-22. Mastigont organelles of T. vaginalis. The pelta (p) encircles the anterior flagella (af), and their kinetosomes (k). A striated rootlet (s) originates from the kinetosome of the recurrent flagellum (krf). (Note the arrangement of the nine triplet microtubules and radiating rootlets (arrow) of the kinetosome.)

Fig. 21. (54,000x).

Fig. 22. (60,000x).

(bar = .1um)

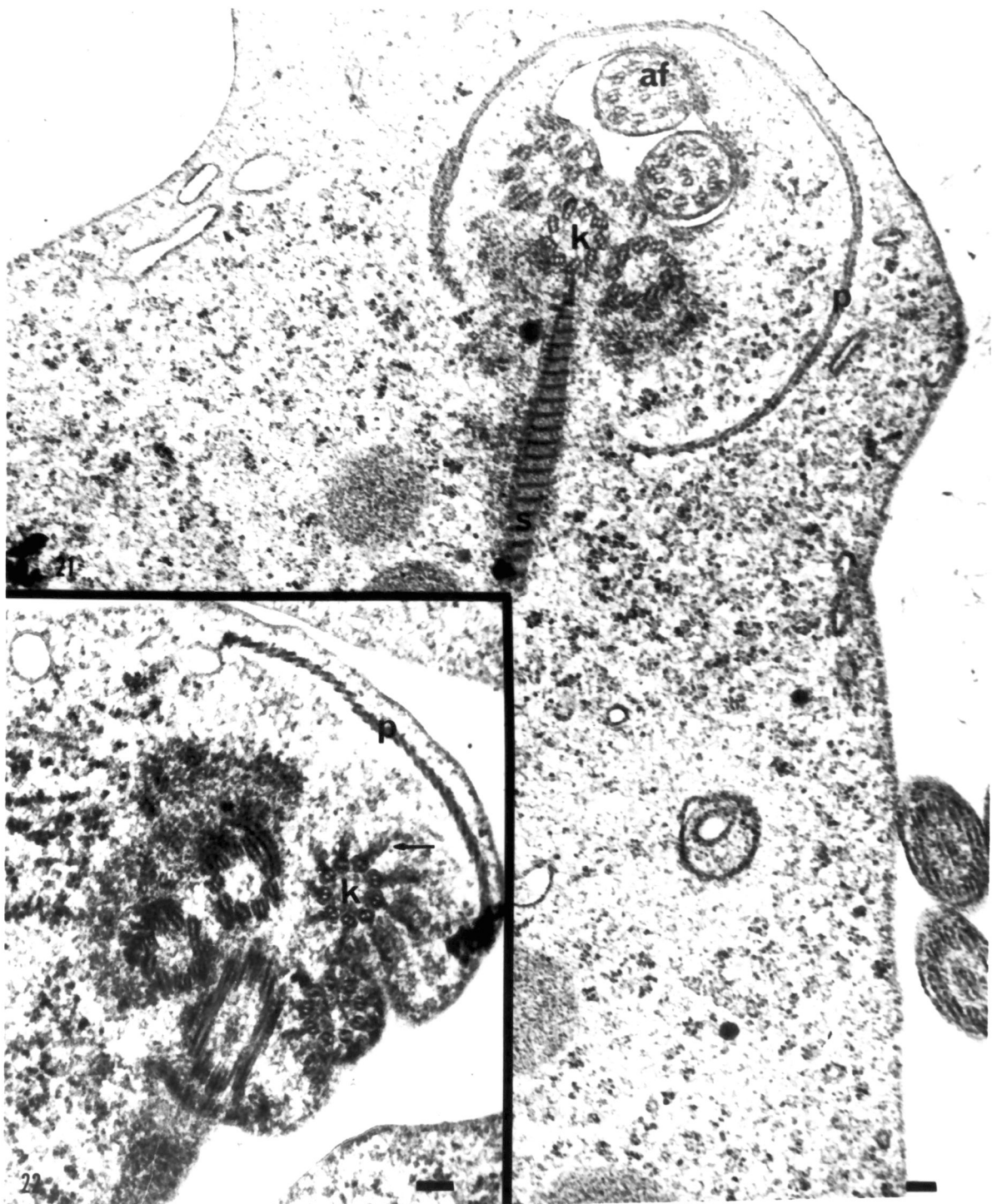


Fig. 23. Cross section of four anterior flagella (af) showing the eukaryotic 9+2 microtubule configuration. (82,500x).

Fig. 24. Cross-section of a recurrent flagellum (rf) associated with the undulating membrane (um). (101,250x).

Fig. 25. A section of T. vaginalis with four anterior flagella (af), recurrent flagellum (rf), and undulating membrane (um) with the marginal lamella (ml) in the undulating membrane. Note the Golgi body (g) and hydrogenosomes (h) in the cytoplasm. (27,500x).

Fig. 26. A longitudinal section of two anterior flagella (af). The basal plate (arrow) is found at the transition from kinetosome (k) to flagellum. Note the microtubules of the pelta (p) and axostyle (a). (74,250x).

(bar = .1 um)

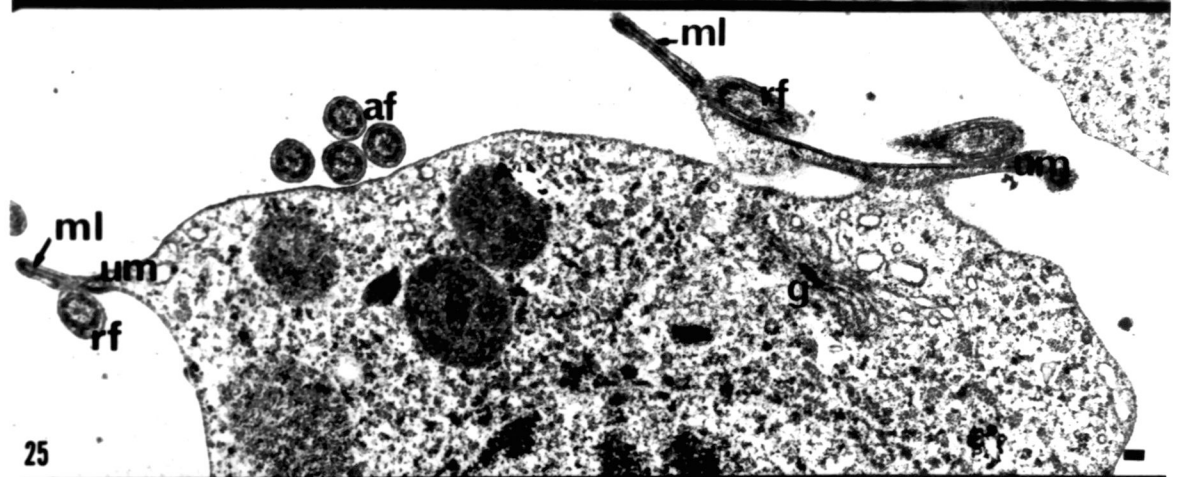
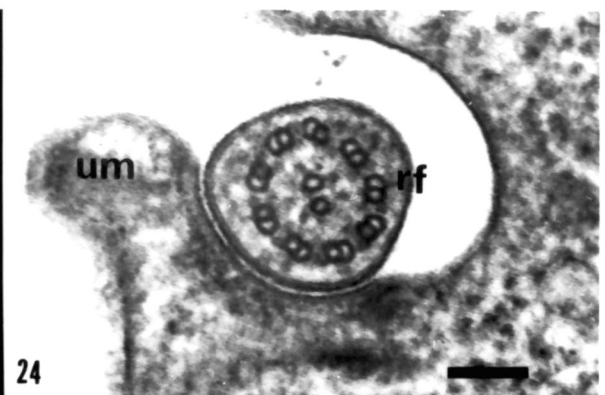
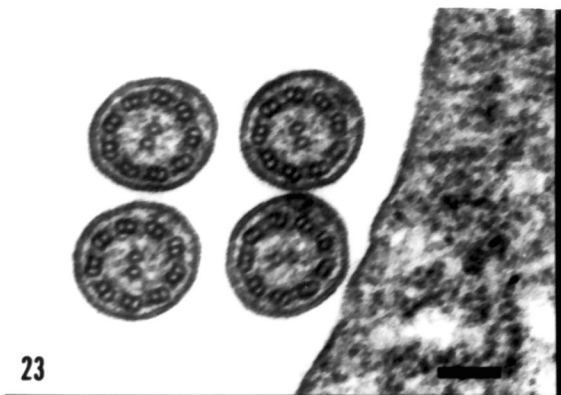


Fig. 27. Longitudinal section of the anterior portion of T. vaginalis. Note the microtubule arrangement in the pelta (p) which has been cut in half. The pelta makes the transition (arrow) into the axostyle (a). (31,500x).

(bar = 1 μ m)

Fig. 28. This section demonstrates the pelta (p) encircling the mastigont organelles. Note the junction (arrow) of the pelta and axostyle. (31,500x).

(bar = 1 μ m)

Fig. 29. Longitudinal section of the axostyle (a) adjacent to the nucleus (n). (24,750x).

(bar = 1 μ m)

Fig. 30. Cross-section of the axostyle (a) and nucleus (n). (54,000x).

(bar = .1 μ m)

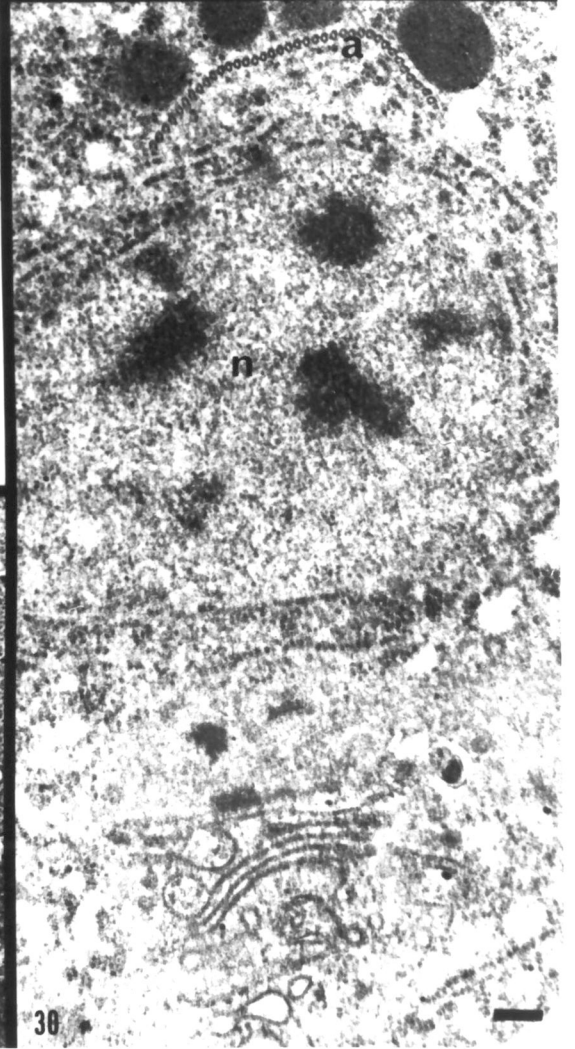
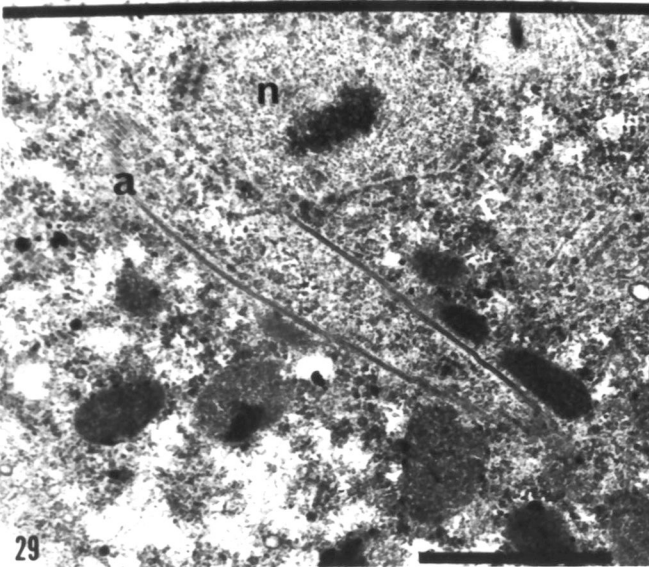
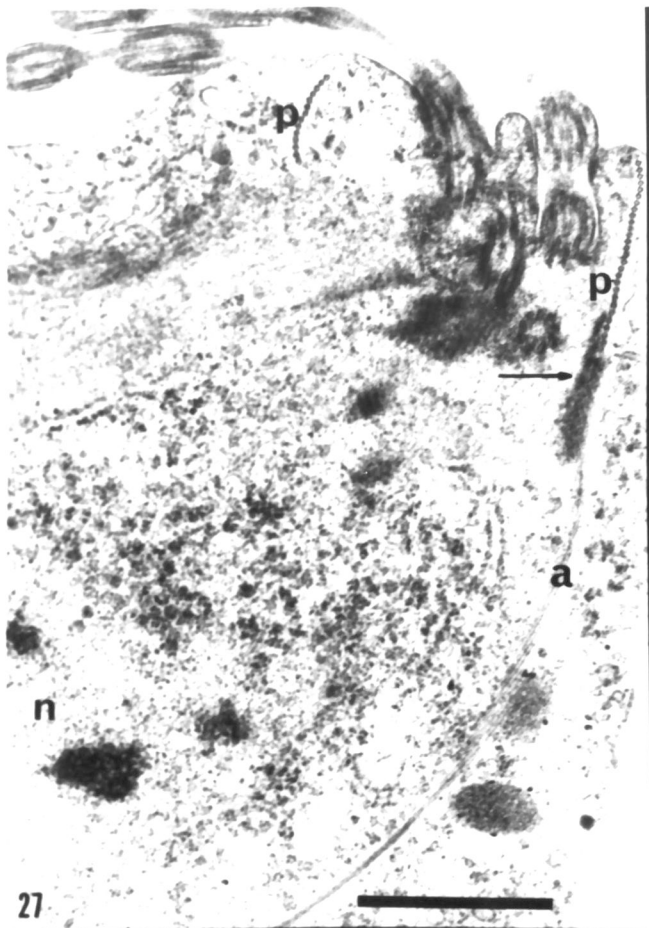


Fig. 31-34. Cross-sections of axostyle (a) progressing from the nuclear region to the caudal portion of T. vaginalis where it becomes more spiral-shaped. The microtubules are connected by a delicate membrane (arrow). Note the hydrogenosomes (h) encircling the axostyle (Fig. 32.

Fig. 35. Longitudinal section of axostyle (a) progressing from the nuclear region to the caudal protuberance. Note the hydrogenosomes (h). (36,000x).

Fig. 31. (36,000x).

Fig. 32. (40,500x).

Fig. 33. (72,000x).

Fig. 34. (54,000x).

(bar = .1 μ m)

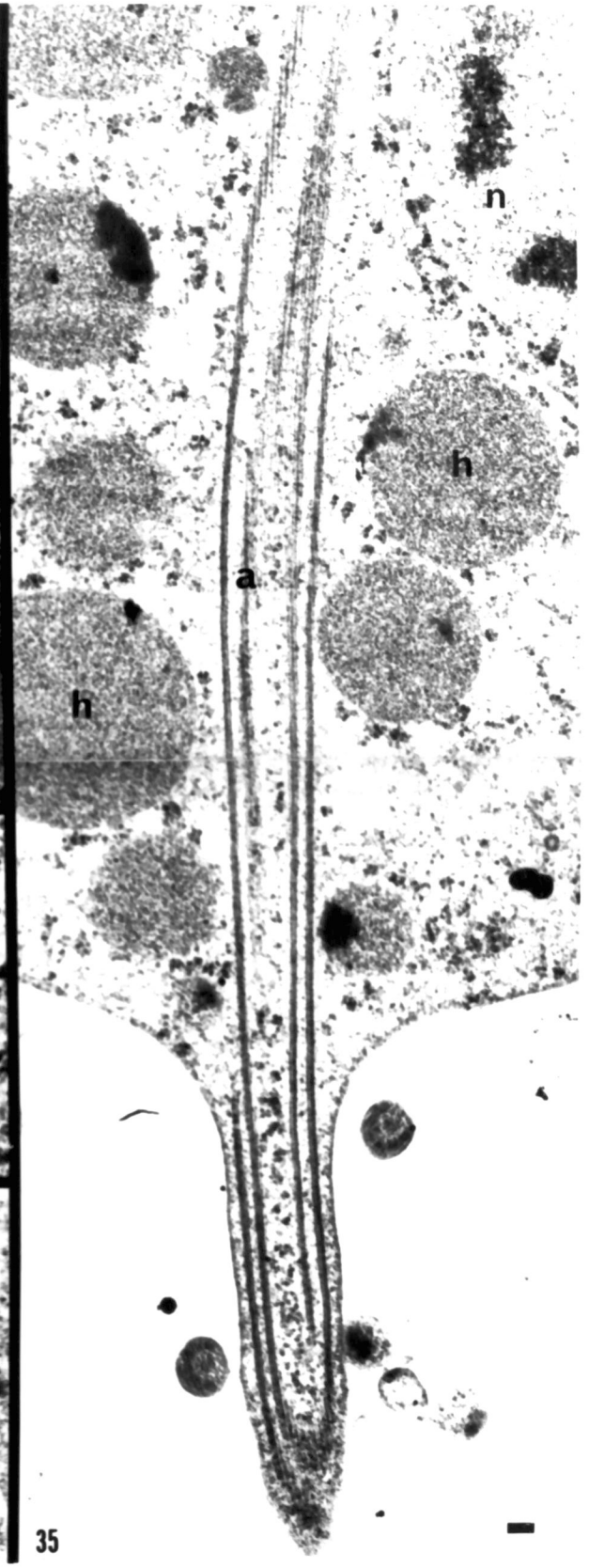
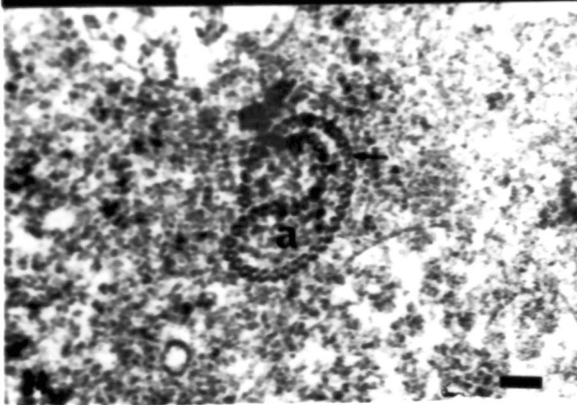
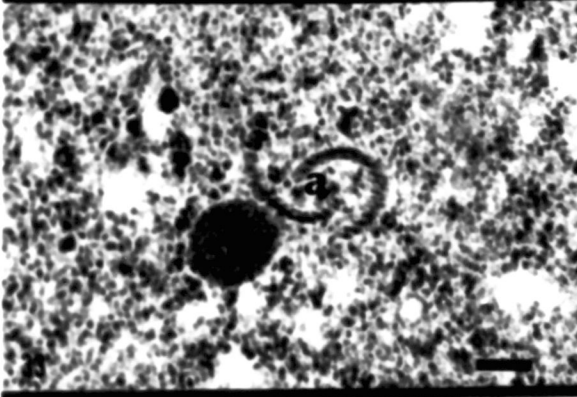
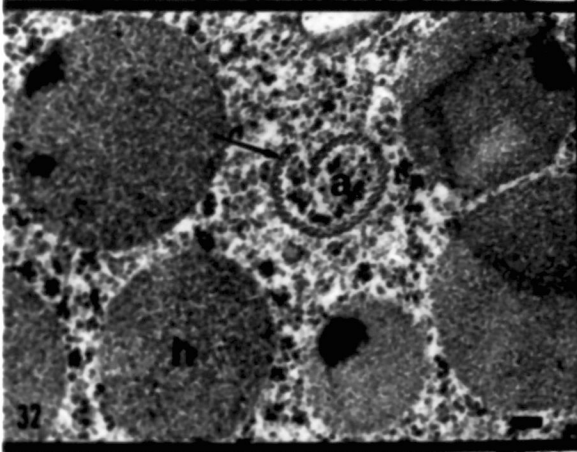
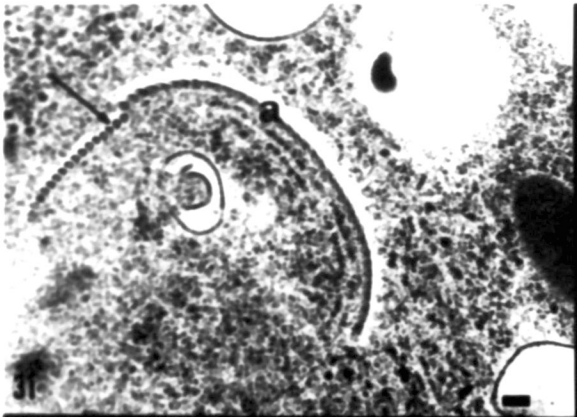


Fig. 36. Longitudinal section through a striated rootlet (s) originating from a kinetosome (k) with a nine triplet microtubule arrangement. (31,200x).

Fig. 37. Longitudinal section of two striated rootlets (s), originating from the bases of two separate kinetosomes (k). (40,000x).

Fig. 38-40. The striated rootlets are slender with alternating dark and light bands.

Fig. 38. (36,000x).

Fig. 39. (58,500x).

Fig. 40. (45,000x).

(bar = .1 μ m)

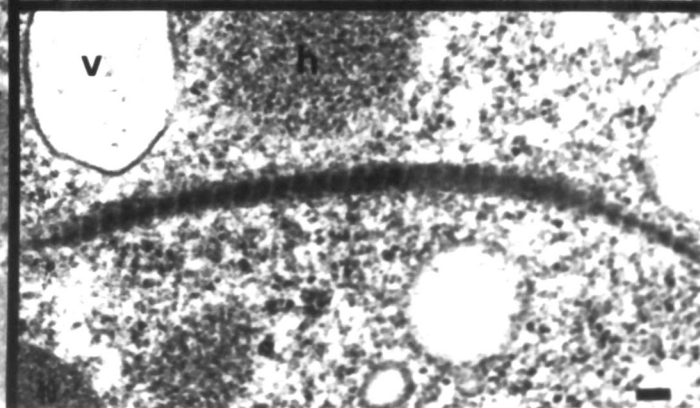
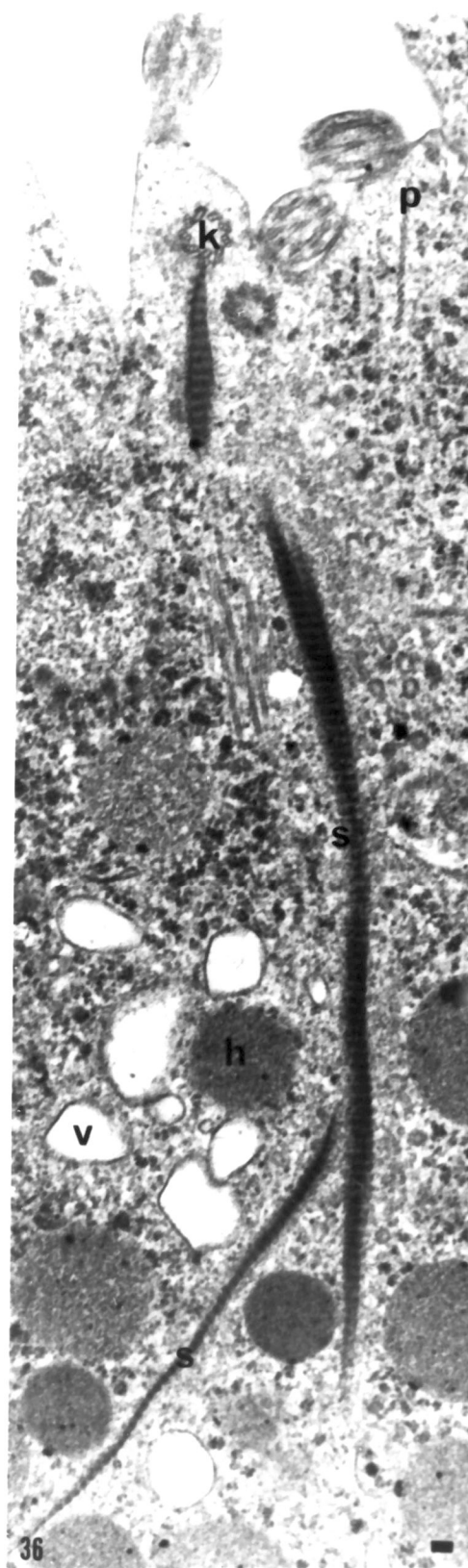


Fig. 41. A Golgi body (g) located beside the nucleus (n). Electron dense material (dm) is often present between the cisternae. The vesicles increase in size as they near the cell periphery. (46,750x).

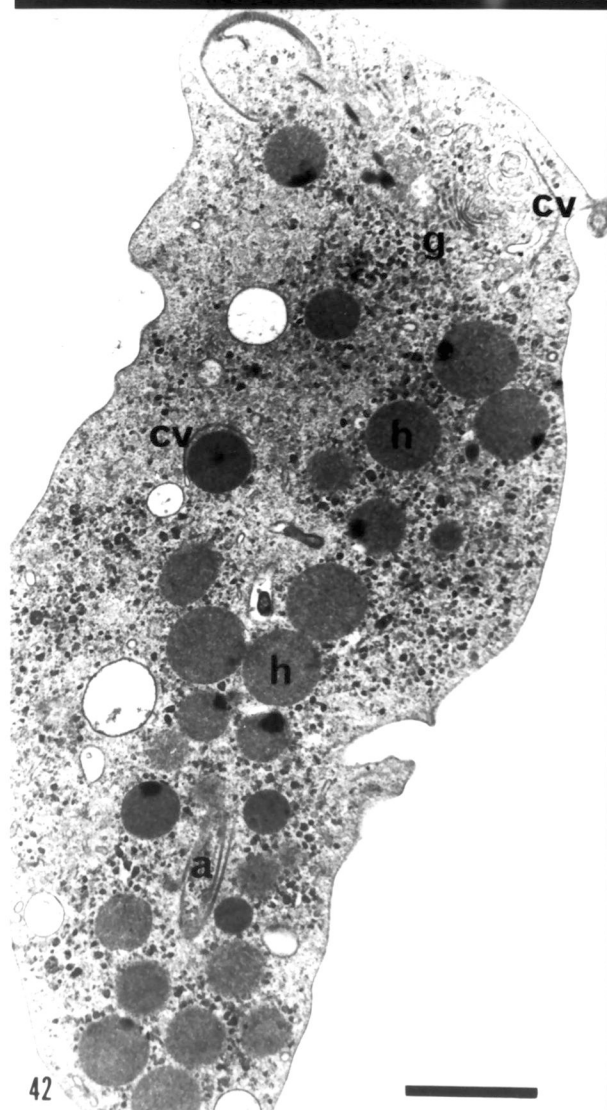
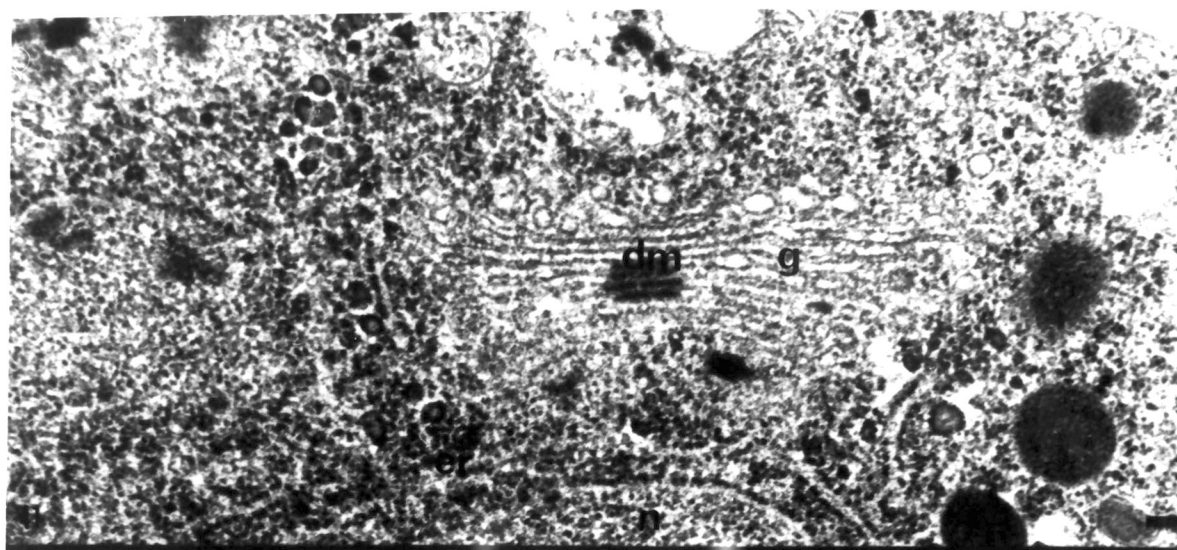
(bar = .1 um)

Fig. 42. Oblique-longitudinal section of T. vaginalis cell. Hydrogenosomes (h) are distributed throughout the cell. Note axostyle (a), Golgi body (g), collapsed vesicles (cv), and vacuoles (v). (17,775x).

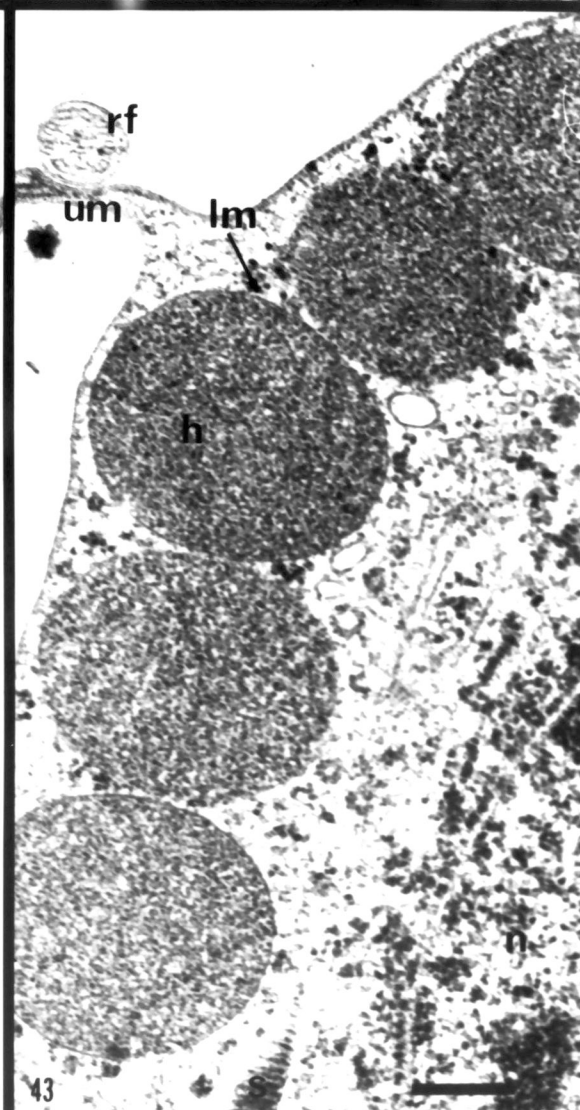
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Fig. 43. Section through hydrogenosomes (h) with limiting membrane (lm). The hydrogenosomes are adjacent to the undulating membrane (um) with its associated recurrent flagellum (rf). (130,000x).

(bar = .1 um)



42



43

Fig. 44. T. vaginalis cell with rosettes of glycogen granules (gg), perinuclear endoplasmic reticulum (er), collapsed vesicles (cv), and vacuoles (v). (33,600x).

Fig. 45. A hydrogenosome (h) with a ribosomal halo (r), and endoplasmic reticulum (er). (45,000x).

Fig. 46. Adhering T. vaginalis cells with glycogen granule rosettes (gg), collapsed vesicles (cv), and vacuoles (v). (45,000x).

Fig. 47. Posterior section with smooth endoplasmic reticulum (er), glycogen granules in rosettes (gg), large vacuoles (v), and hydrogenosomes (h). (27,500x).

(bar = .1 um)

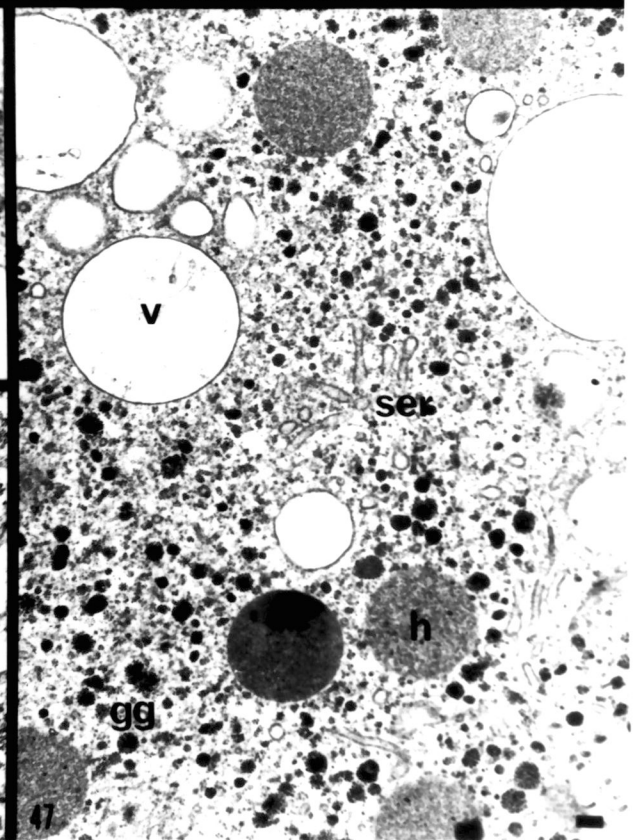
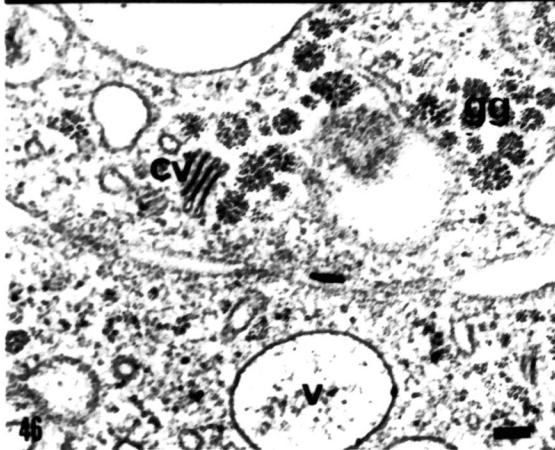
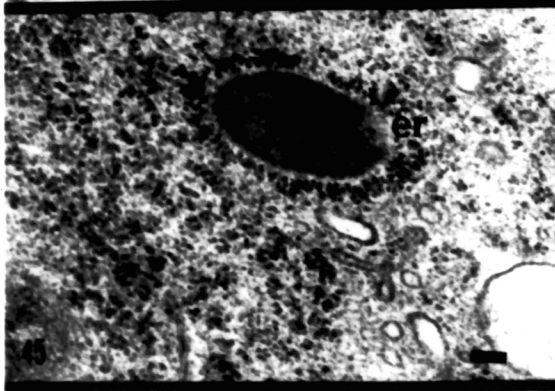
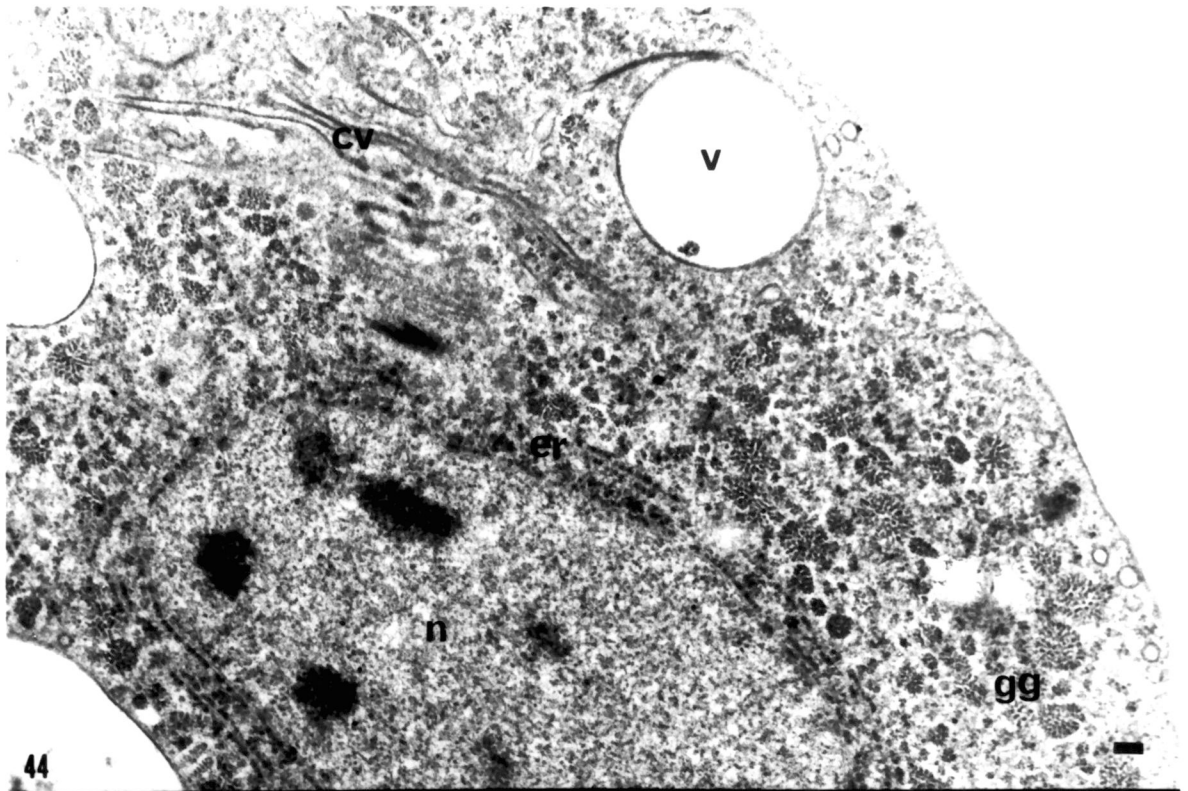


Fig. 48-49. SDS-Polyacrylamide gel electrophoresis (PAGE) analysis of protein in whole cell T. vaginalis homogenates of AA and OM.

Fig. 48. 7.5% SDS-Polyacrylamide gel electrophoresed 20 hrs at 5 MA. Protein concentrations: Lane 1 (3ug), lane 2 (2.7ug), lane 3 (6ug), lane 4 (5.4ug), lane 5 (9ug), lane 6 (8.1ug), lane 7 (12ug), lane 8 (10.8ug), lane 9 (15ug), lane 10 (13.5ug). Lane 11 BSA (10ul). Lane 12-SDS 6 (5ul). Lane 13-SDS 7 (5ul). Lane 14-15 (unknown). AA lanes 1, 3, 5, 7, 9. OM lanes 2, 4, 6, 8, 10.

Fig. 49. 15% SDS-Polyacrylamide gel electrophoresed 20 hrs at 10 MA. Protein concentrations: Lanes 1-2 (3ug); 3-4 (6ug); 5-6 (9ug); 7-8 (12ug); 9-10 (15ug). Lane 11-SDS 6 (5ul). Lane 12-SDS 7 (5ul). Lane 13-BSA (10ul). OM lanes 1, 3, 5, 7, 9. AA lanes 2, 4, 6, 8, 10.

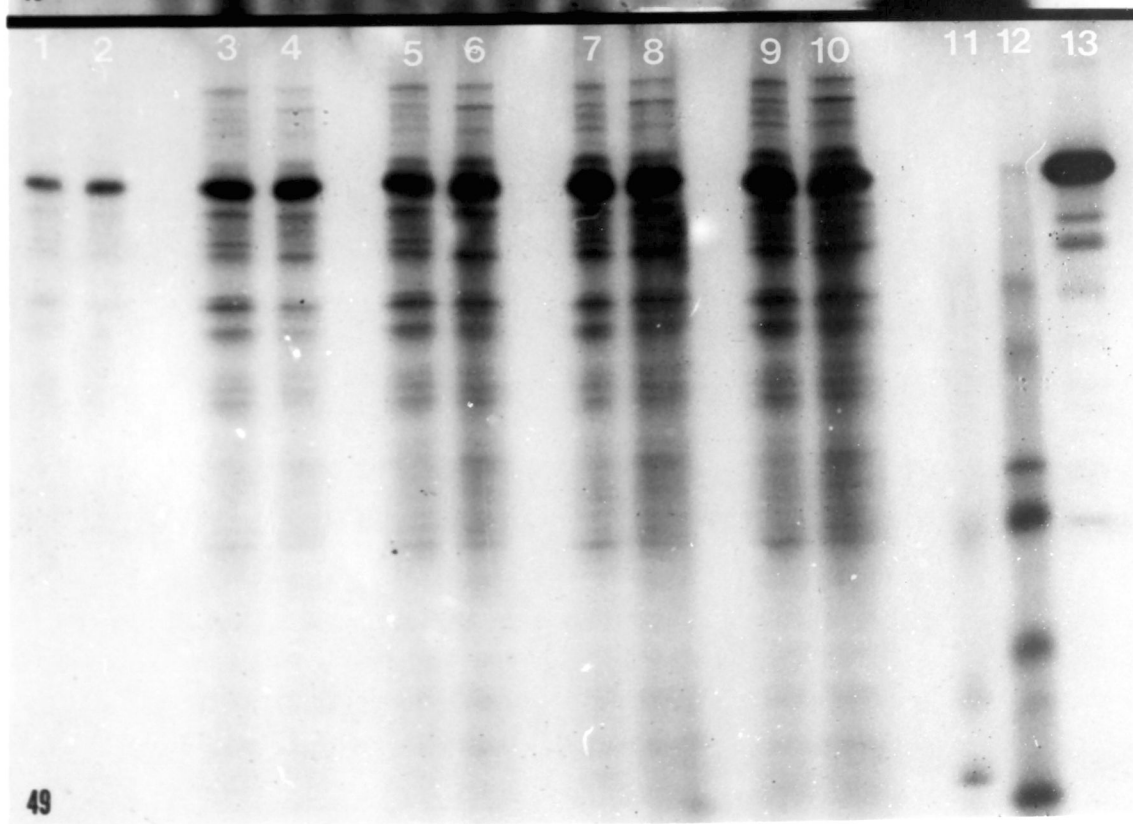
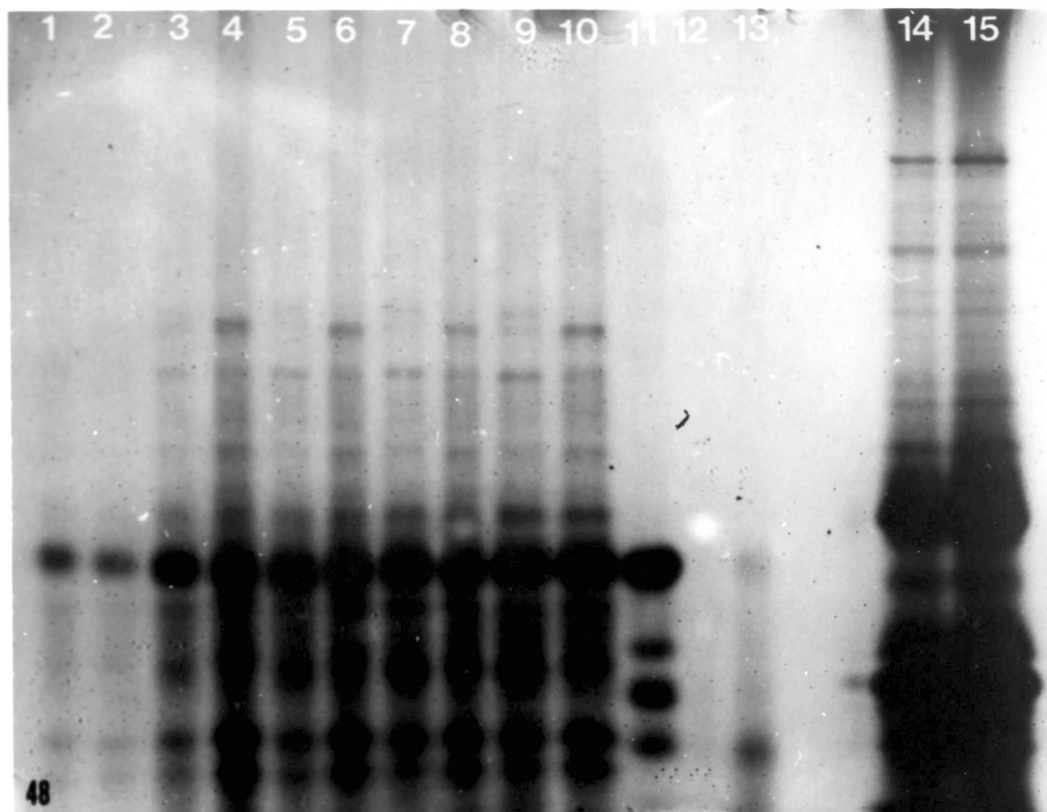


Fig. 50. Lactic acid production (meq/100mls) of AA and OM strains in media (+CHO and -CHO) at 48 hrs and 7 days.

Lactic Acid (meq/100 ml)

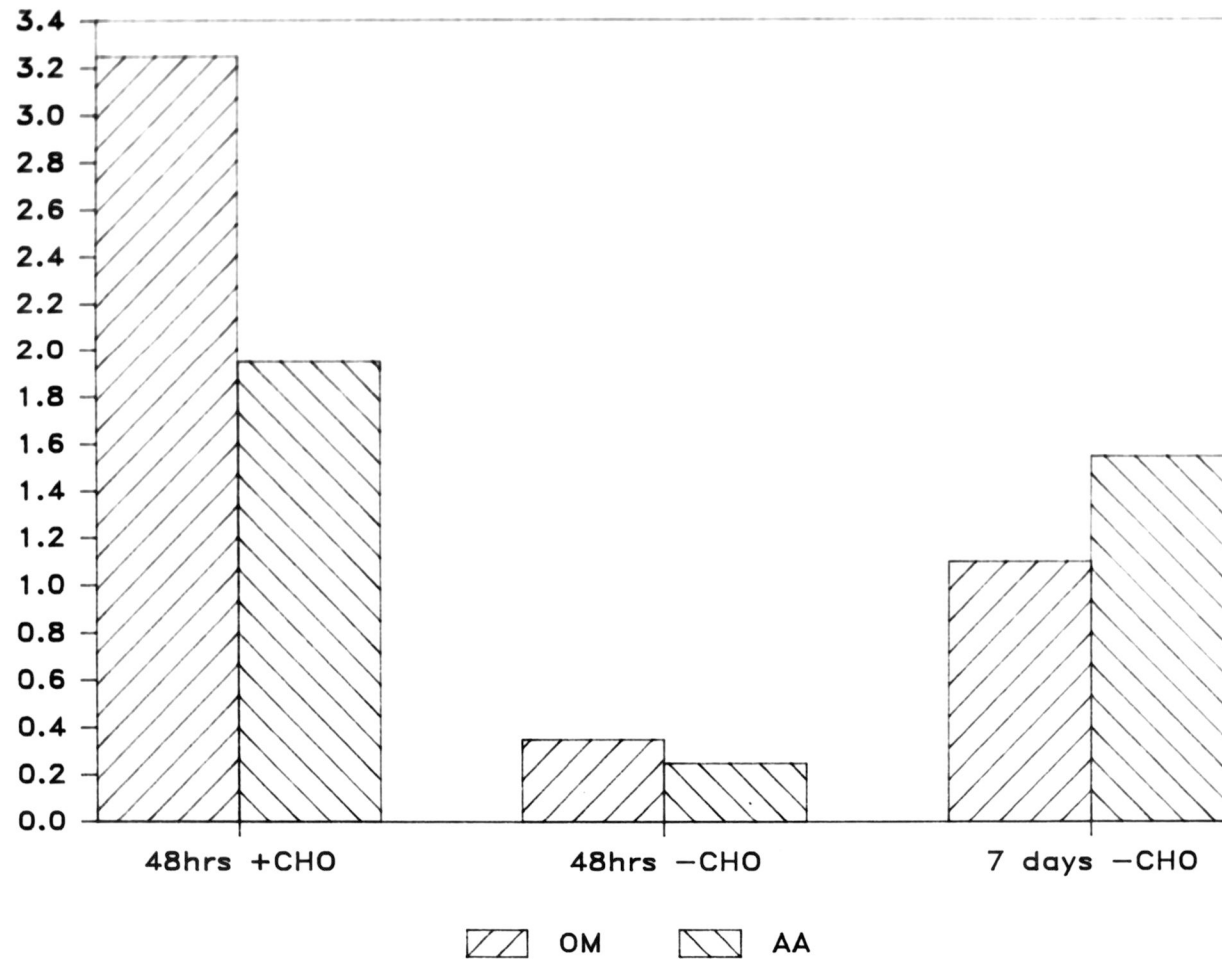


Fig. 51. Diagrammatic surface and internal representations of OM strains. Marginal lamella (ml), glycogen granules (gg), axostyle (a), pelta (p), nucleus (n), Golgi body (g), kinetosome (k), anterior flagella (af), recurrent flagellum (rf), undulating membrane (um), hydrogenosome (h), and food vacuoles (v).

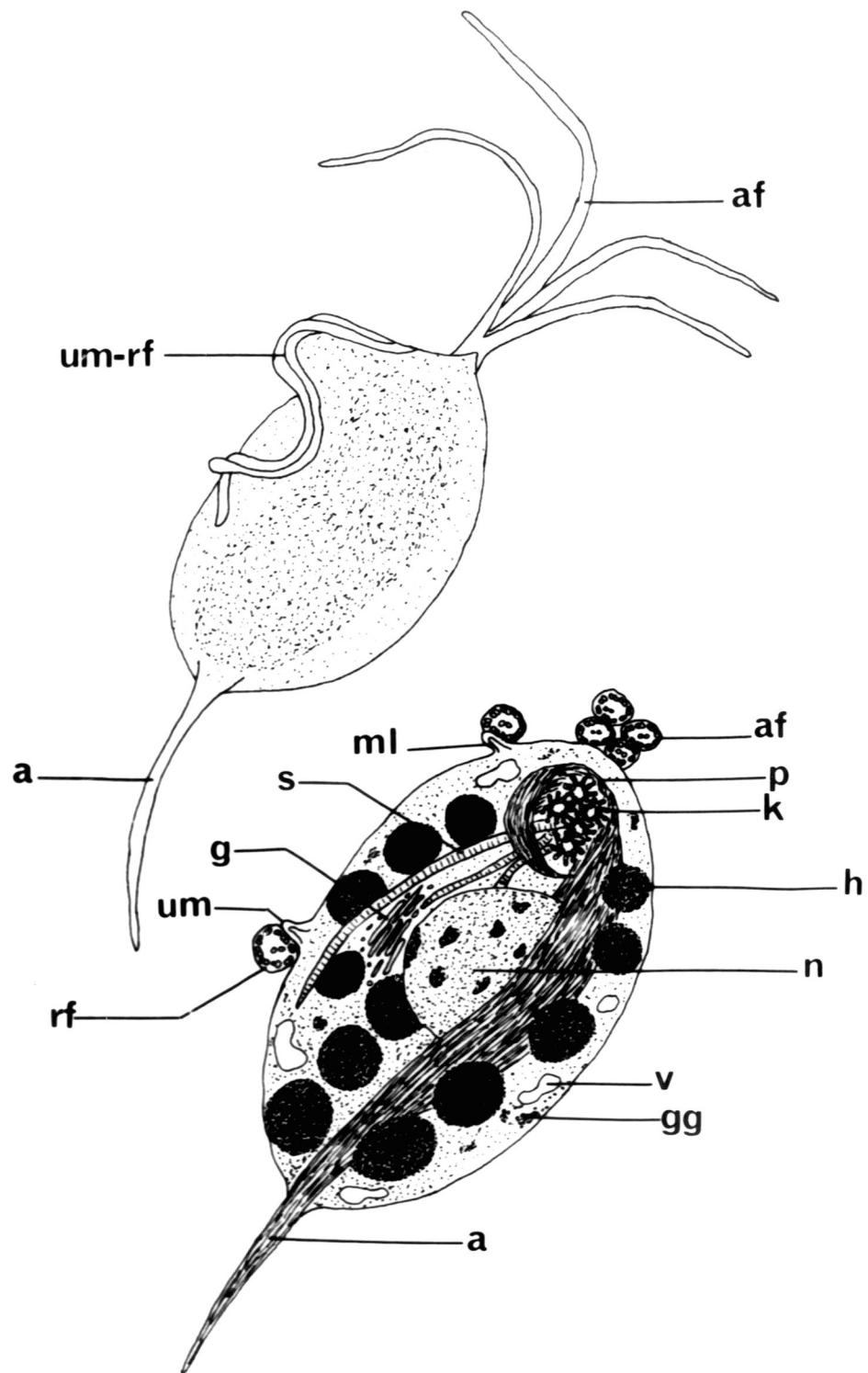
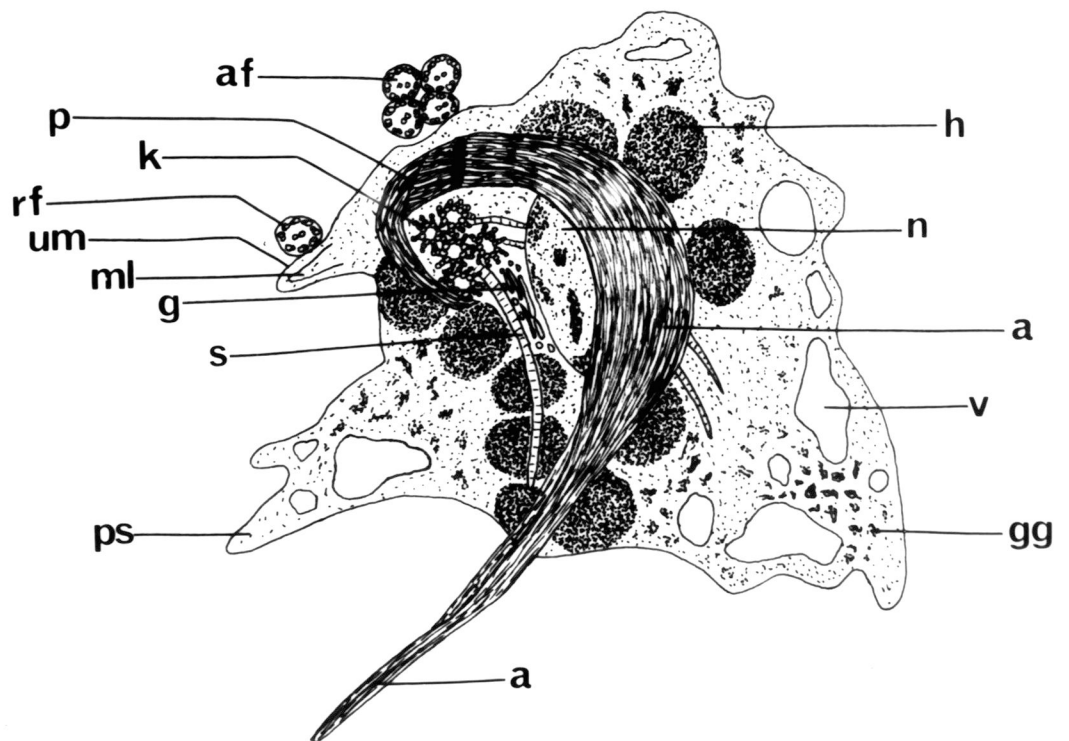
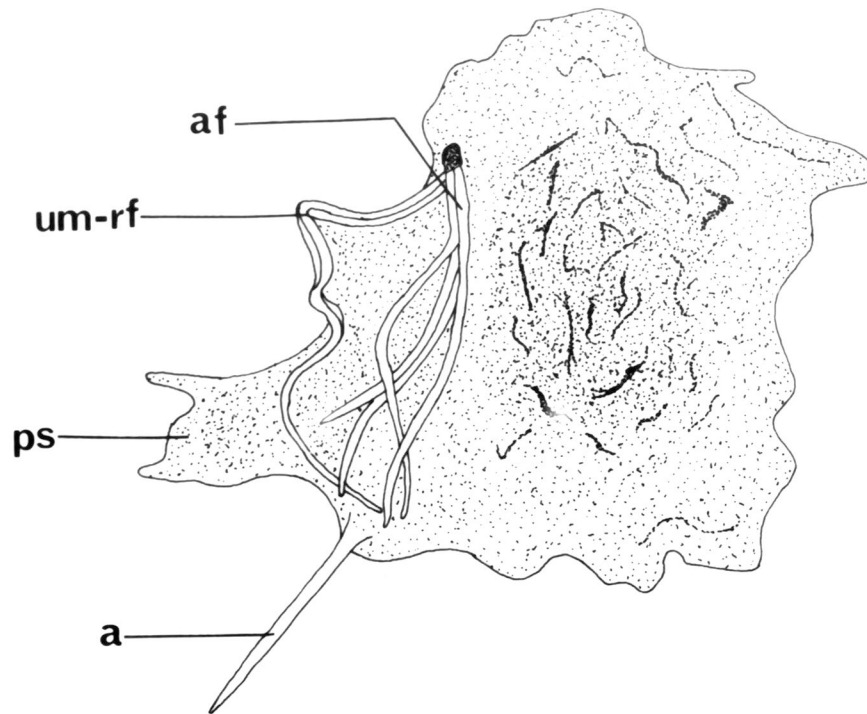


Fig. 52. Diagrammatic surface and internal representation of AA strains. Marginal lamella (ml), glycogen granules (gg), axostyle (a), pelta (p), nucleus (n), Golgi body (g), kinetosome (k), hydrogenosome (h), vacuoles (v), anterior flagella (af), recurrent flagellum (rf), undulating membrane (um), pseudopodia (ps).



DISCUSSION

CULTURE MEDIUM

After testing a variety of media, the medium found to best support growth of T. vaginalis was a variation of Diamond's TYM medium. Diamond (1957) incorporated both L-cysteine hydrochloride and L-ascorbic acid as reducing agents. Cysteine was omitted in the present study because ascorbic acid alone was found to produce a preferred oxidation-reduction potential (Hollander and Frost, 1965). The addition of vitamin B¹² (Hollander and Leggett, 1985), and the substitution of tapwater for distilled water were found to improve growth rate and strain morphology. We can only speculate that this is due to unspecified trace minerals found in the water supply. Originally, glutamine was included also, but after a 6-week experiment excluding glutamine from the medium, it was found that the simplified medium supported populations equal to those in the original medium. Over 25 serial transfers were maintained without visible differences in shape, size, or motility.

The accumulation in culture medium of lactic acid from metabolized carbohydrates dictates a short life span for T. vaginalis. In vivo, a normal pH value for vaginal secretions is 4.5 or less due to the high level of lactic acid from glycogen (Hanna et al., 1985). When T. vaginalis is present, secretions range from 6.0 to 7.5. Optimum growth for this organism in culture occurs in media with an initial pH range of 5.8-6.8 (Lumsden et al., 1966). In this study, the pH of both (+CHO, -CHO) was routinely found to be 6.8 without adjustment. Following inoculation, the medium became more

acidic because of the lactic acid build-up from carbohydrate metabolism. Preliminary studies showed that an adjustment to a more basic pH (7.4) increased the logarithmic phase, but this initial pH supported growth for a shorter period of time. This was due to the increased rate of lactic acid production, and therefore increased toxicity level to T. vaginalis. This medium would have required more frequent culture transfers than the +CHO medium with a pH of 6.8.

In comparison with the +CHO medium, the -CHO medium sustained growth for a longer period of time. The absence of a lactic acid build-up from maltose metabolism enabled growth for up to four weeks. Even though maltose was absent, the medium still had a carbon source from the glucose present in the bovine serum and yeast extract (Vazquezdelara-Cisneros and Arroyo-Begovich, 1984). Therefore, the -CHO medium did accumulate some lactic acid over a period of 7 days. The organisms in the -CHO medium were less active but retained their morphology. The OM strain was more active than the AA form possibly metabolizing more carbohydrates thus producing more lactic acid.

COLONIAL MORPHOLOGY

When observing pour plate colonies microscopically there was no correlation between strain (OM, AA) and colony size or shape. The colonies varied in size because of their location in the medium. If colonies were located between the glass and the media, they tended to spread considerably. Whereas if the colonies were located within the medium they were not so large because of the tension from the medium. Another variable to consider was the distance between the surface of the medium and the colony. This

distance can create an illusion of the sharpness of colony margins.

There was some variation when colonies of pure strains were observed in culture tubes. The OM strains had diffuse growth throughout the medium except for the uppermost portion which has a higher oxygen content T. vaginalis does not prefer. AA strains formed small pinpoint colonies in the medium, because of their inability to be motile and also from their tendency to form clusters.

CELL MORPHOLOGY

Abnormal forms of T. vaginalis have been reported previously (Powell, 1936; Trussell, 1947; John and Squires, 1978; Buckner and Mikel, 1983). The ability to vary size and shape has been attributed to environmental conditions, degeneration, and growth phase (Honigberg and King, 1964). The OM forms were often seen attached to glass slides while feeding. They also altered their shape temporarily by creating extensions of cytoplasm, then reverted back into their normal ovoid shape. These temporary alterations were characteristic of the OM strain. The AA forms differ in that they were not temporary. They were nonmotile and had inactive or absent flagella. These forms would adhere at one location, unless disturbed. In this area the cells would feed and reproduce to form close colonies.

Since when the AA and OM strains were cultured in +CHO medium, they retained their morphology, the theory that amoeboid tendencies are degenerative and due to poor nutrition is unsupported. The OM and AA forms were both stable populations when grown in culture.

In an effort to disclose any fine structural differences, the AA and OM forms were compared via electron microscopy. Except for cell shape, the two

forms were found to exhibit similarities in organelle structure and arrangement.

Nielsen and Nielsen (1975) described a network of cytoplasmic microfilaments in pseudopodia present in amoeboid T. vaginalis from patient biopsies. Nielsen (1975) described also a filamentous cell coat in in vivo specimens. Both the microfilaments and cell coat characteristics were associated with virulent T. vaginalis. Due possibly to fixation technique or the amount of time cultures were kept in vitro, these structures were not present in the organisms in this study.

Cappuccinelli and Varesio (1975) were interested in the adhesive tendencies of T. vaginalis. Their studies with cytochalasin B to inhibit intracellular microfilaments related optimal adhesion of T. vaginalis to glass with the presence of peripheral pseudopodia mediated by contractile microfilaments. Therefore, the adhesive processes of the AA form would seem to be a likely place to find microfilaments. Further studies could conclude this.

AMOEBO-FLAGELLATES

Amoeb-flagellates are a diverse group of organisms which at some point in their life cycle have both amoeboid and flagellated forms (Fulton, 1977). These temporary transformations may be flagellate-to-amoeba or amoeba-to-flagellate. These organisms are placed among the Protozoa in either the Sarcodina or Mastigophora classes. The amoeb-flagellates are thought by some to be an evolutionary midpoint between amoebae and flagellates (Fulton, 1970). The two forms (OM and AA) of T. vaginalis described may have a similar connection. The more prominent OM form may have "evolved" from the

AA form or the OM form may have regressed into the AA form.

The protein bands in the SDS-PAGE were similar enough to conclude that OM and AA forms were of the same species. However, in some of the gels, bands were absent in the AA strains. This suggests some protein variation, probably in the surface proteins (Alderete et al., 1986). To prove this, further investigations would be necessary.

PATHOGENICITY

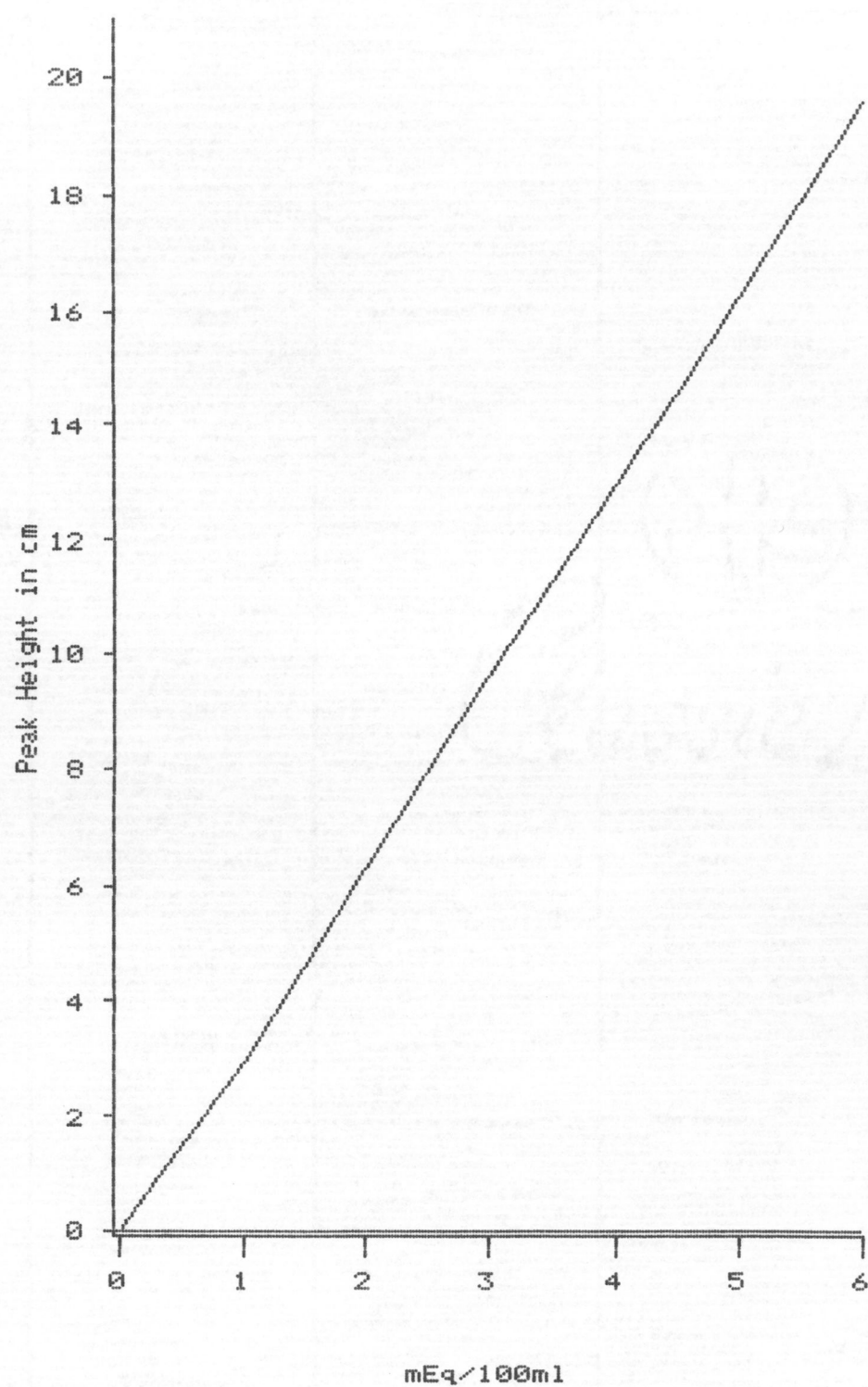
Pathogenicity has been related to growth rate (Kulda et al., 1970; Honigberg, 1961), and amoeboid movements or adherence (Rasmussen et al., 1986). The isolation in this study of a stable amoeboid strain provides an excellent opportunity to determine the relationship between cell morphology and a pathogenicity test.

FURTHER STUDIES

Since this is a preliminary report describing AA as a variant form of T. vaginalis, further studies would reinforce this theory. The following could be helpful:

1. a pathogenicity test using the mouse-assay to compare OM and AA strains.
2. isolation and identification of protein bands present in OM but absent in AA.
3. treatment of AA and OM with cytochalasin B and observing the ultrastructure, especially the intracellular microtubules.

LACTIC ACID STANDARD CURVE



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